

**Associative Phase Separation in Admixtures of Pea Protein Isolates with Gum  
Arabic and a Canola Protein Isolate with  $\iota$ -Carrageenan and Alginate**

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## ABSTRACT

The overall goal of this thesis is to better understand mechanisms governing associative phase separation within admixtures of plant proteins (e.g., pea and canola) and anionic polysaccharides (e.g., gum Arabic, alginate or  $\iota$ -carrageenan). The process involves the electrostatic attraction between two biopolymers of opposing charges, and typically results in the formation of both soluble and insoluble complexes during an acidic pH titration. If successful, polysaccharides could be triggered to coat the protein's surface to give novel, and hopefully improved functionality as ingredients for food and biomaterials.

In the first study, the effect of protein enrichment and pH on the formation of soluble and insoluble complexes in admixtures of pea legumin (Lg) and vicilin (Vn) isolates with gum Arabic (GA) was investigated by turbidimetric, surface charge and fluorometric measurements. The solubility of the protein isolates and mixed biopolymer systems was also studied as a function of pH. Enrichment of the crude Lg and Vn isolates by low pressure liquid chromatography led to a shift towards higher pHs at the onset of soluble complex formation in the presence of GA for both protein isolates, whereas the onset of insoluble complex formation was unaffected. Complexation of the Lg (or Vn) isolates with GA resulted in a shift in the pH where neutrality (zeta potential = 0 mV) occurred to lower pH values, relative to the Lg (or Vn) isolates alone. In the case of the enriched Vn isolate, changes to its tertiary structure were observed by fluorometry upon complexation with GA, whereas no changes were found for the enriched Lg isolate. Complexation of Lg and Vn isolates with GA also had little effect on their solubilities relative to protein alone solutions.

In the second study, the formation of soluble and insoluble complexes, and the nature of their interactions as determined by optical density analysis, were investigated in admixtures of canola protein isolate (CPI) and anionic polysaccharides (alginate and  $\iota$ -carrageenan) as a function of pH and biopolymer weight mixing ratio. The solubilities of formed complexes were also investigated versus protein alone. In both CPI-

polysaccharide systems, critical pH associated with the onset of soluble and insoluble complexes shifted to higher pHs as the mixing ratios increased from 1:1 to 20:1 (CPI:polysaccharide), and then became constant. These complexes formed primarily through electrostatic attractive forces with secondary stabilization by hydrogen bonding. The solubilities of the CPI-alginate complexes were significantly enhanced relative to CPI alone or CPI- $\kappa$ -carrageenan, which were similar.

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## LIST OF ABBREVIATIONS AND SYMBOLS

### ABBREVIATIONS

ANOVA	analysis of variance
BSA	bovine serum albumin
cm	centimeters
Cp	total polymer concentration
CPI	canola protein isolate
DEAE	diethylaminoethyl
e.g.	for example
FI	fluorescence intensity
g	grams
<i>g</i>	force of gravity
GA	gum Arabic
GDL	glucono delta lactone
GMO	genetically modified organism
h	hours
i.e.	that is
kDa	kiloDaltons
kg	kilograms
L	liters
Lg	legumin
M	molar
mg	milligrams
mL	milliliters
min	minutes
mM	millimolar
mV	millivolts

nm	nanometers
PAGE	polyacrylmide gel electrophoresis
PF	pea flour
pH <sub>c</sub>	pH at which soluble complexes form
pH <sub>φ1</sub>	pH at which insoluble complexes form
pH <sub>φopt</sub>	pH where maximum coacervate formation occurs
pH <sub>φ2</sub>	pH at which complexes dissociate
pI	isoelectric point
PPI	pea protein isolate
S	sedimentation coefficient
SDS	sodium dodecyl sulphate
UV	ultraviolet
V <sub>n</sub>	vicilin
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

## SYMBOLS

$\alpha$	alpha
$\beta$	beta
°C	degrees Celsius
$\iota$	iota
$\kappa$	kappa
$\lambda$	lambda
$\mu\text{g}$	micrograms
$\mu\text{L}$	microlitre
$\mu\text{M}$	micromolar
$\mu\text{S}$	microsiemens
%	percent
$\phi$	phi
$\pm$	plus/minus

## CHAPTER 1.0 INTRODUCTION

### 1.1 Summary

Protein ingredients used by the food industry represent a multi-billion dollar industry presently dominated by animal proteins, such as gelatin, ovalbumin, casein and whey. In 2011, the US protein ingredient market for food is forecasted to be worth ~\$2.84 billion, with ~\$1.17 and ~\$1.67 billion coming from plant and animal sources, respectively (BCC Research, [www.bccresearch.com](http://www.bccresearch.com)). With increased concerns over the safety of animal-derived products, growing dietary preferences and consumer demand for healthier foods; market trends are shifting towards lower cost and plant-based alternatives (e.g., soy). However, despite plant proteins experiencing greater market growth than animal-derived ingredients, their wide spread use has been hindered by their reduced functionality (e.g., solubility) relative to animal-based products and their distinct flavours. With the exception of soy, plant proteins are now primarily used to fortify feed. Over the past few decades, extensive effort has been made to improve plant protein functionality through chemical (Paulson and Tung, 1998; Schwenke et al., 1998) or enzymatic (Chabanon et al., 2007; Wu and Muir, 2008) means, however their use in foods and biomaterials has still been limited. An alternative approach is to create blended biopolymer ingredients by complexing plant proteins with polysaccharides through a process known as associative phase behaviour (or complex coacervation). The resulting complexes could have a positive impact on functionality and food structure relative to the protein alone, and in principle, could be tailored for specific applications in the food industry.

Complex coacervation involves inducing an electrostatic attraction between biopolymers (e.g., proteins and polysaccharides) of opposing charges (Doublier et al., 2000). Depending on the biopolymer characteristics (e.g., type, reactive groups, chain length, branching, flexibility, hydrophobicity, mixing ratio and concentration) and solvent conditions (e.g., pH and salts), varying degrees of interactions can ensue,



leading to the formation of stable liquid coacervates (i.e., complexes with entrapped solvent) or in some cases precipitates (Schmitt et al., 1999; Doublier et al., 2000). By controlling these conditions, polysaccharides can be triggered to coat (or bridge) individual or aggregated protein(s) to create novel complexes (ingredients). Associative phase separation has been well studied in the literature, primarily involving proteins contained within milk such as bovine serum albumin (BSA) (Wang et al., 1996; Wen and Dubin, 1997), casein (Syrbe et al., 1998),  $\beta$ -lactoglobulin (Sanchez and Renard, 2002; Harnsilawat et al., 2006) and whey (Weinbreck et al., 2004a,b) with anionic polysaccharides. An understanding of complex formation involving admixtures of plant proteins is more limited, probably due to their heterogeneous structure and reduced solubility relative to animal proteins. As examples, mixtures of canola protein isolate and alginate (Arntfield and Cai, 1998), soy protein isolate and carrageenan (Ortiz et al., 2004), wheat  $\alpha$ -gliadin and gum Arabic (Chourpa et al., 2006), and pea protein isolate and gum Arabic (Liu et al., 2009) have been studied. Although mechanisms underlying complex formation are not fully elucidated, they are widely believed to be associated with two pH-dependent structure-forming events, corresponding to the formation of both soluble and insoluble complexes (Girard et al., 2004).

The present research builds upon current knowledge of mechanisms governing complex coacervation involving pea proteins and gum Arabic (GA) polysaccharides, and initiates new work on structure-function relationships involving mixtures of canola proteins with sulphated ( $\kappa$ -carrageenan) and carboxylated (alginate) polysaccharides during complex coacervation. A better understanding of complexation in admixtures may lead to increased demand and utilization of pea and canola protein products in foods and biomaterials. Both pea and canola represent industrially important crops to Saskatchewan, which are currently underutilized based on their protein content and value. In 2009, approximately 2.6 and 5.7 million metric tonnes of pea and canola, respectively were produced in Saskatchewan (Saskatchewan Ministry of Agriculture, 2009). Although pea protein products are commercially available, their use has been hindered by their functionality (e.g., solubility, gelation) and flavour. In contrast, canola protein products have recently received GRAS status (generally recognized as safe) by the food industry, and should reach the marketplace in the near future.

Field peas (*Pisum sativum*) are dominated by two major globulin proteins: legumin (Lg; 350-400 kDa) and vicilin (Vn; 150 kDa) (Ducel et al., 2004). In contrast, GA is a heterogeneous anionic carboxylated polysaccharide derived from the exudates of *Acacia senegal* and *Acacia seyal* trees. Mechanistic studies of pea protein-GA mixtures under conditions favouring associative phase separation have been limited. Previous work by Ducel et al. (2004) on pea globulin-GA complexation involved studying solvent and biopolymer effects under conditions showing maximum protein-polysaccharide interactions, and their potential as an encapsulating agent for entrapping oils. Liu et al. (2009) studied the effect of pH, salt concentration and biopolymer mixing ratio on complex formation in admixtures of a pea protein isolate and GA. The authors found optimal conditions for complex formation occurred at a 2:1 pea protein isolate-GA mixing ratio in the absence of added salt and a pH of 3.60. Liu et al. (2009) created phase diagrams describing biopolymer and salt effects on critical structure-forming events (i.e., the formation of soluble and insoluble complexes). The authors also identified the importance of protein-protein aggregation during complex formation. The functional behaviour (solubility, emulsifying and foaming properties) of pea protein isolate-GA complexes was also studied by Liu et al. (2010b), to find emulsion and foam stability to be improved relative to the protein alone, whereas foam expansion was unaffected by the presence of GA. The pH-solubility minimum was found to be broadened relative to PPI, and shifted towards more acidic pHs. Complex formation between pea protein isolate and GA was also found to be driven by electrostatic attractive forces between oppositely charged biopolymers, with secondary stabilization by hydrogen bonding (Liu et al., 2010a). Hydrophobic interactions were found to enhance complex stability at lower pH (pH 3.10), but did not play a role in complex formation. Chourpa et al. (2006) investigated the conformational modifications to a pea globulin isolate during complex coacervation with GA as a function of pH, by Raman microspectroscopy. The authors found that the globulin-GA complexes at pH values between 4.0-3.5 favoured both  $\alpha$ -helical and random coil regions, and had little or no effect on the  $\beta$ -sheet content. While at lower pH values (pH 2.75) an increase in  $\beta$ -sheet formation was seen with a decrease in randomness. Each of these studies were deficient with respect to understanding the role of the major globulin fractions (Lg and Vn) on:

the formation of soluble and insoluble complexes, associated changes to protein structure, and the resulting effects on complex functionality.

Canola is dominated by two main storage proteins: cruciferin (globulin) and napin (albumin). In contrast, alginate is a linear polyuronic polysaccharide extracted from brown seaweed (*Phaeophyceae*), whereas  $\iota$ -carrageenan is a linear sulphated polysaccharide extracted from red seaweed (*Rhodophyceae*). The published work on the study of plant proteins and highly charged polysaccharides is limited. Arntfield and Cai (1998) investigated phase behaviour in canola-polysaccharide systems at high total biopolymer concentrations (1-1.5% w/w) at solution pH where biopolymers had opposing charges. They found that mixtures of neutral polysaccharides (e.g., methylcellulose, guar gum) were incompatible with the canola proteins, whereas interactions occurred with charged polysaccharides (e.g., pectin and alginate) below the isoelectric point (pI) of the protein. From a review of literature, mechanistic studies describing complexation between canola proteins and charged polysaccharides in dilute aqueous solutions has yet to be undertaken, despite canola's potential as a plant protein ingredient.

## 1.2 Hypotheses

The central hypothesis of this thesis is that the functional attributes of both pea and canola isolates can be improved by controlling the coacervation process and the resulting structures.

This research tested the following five main hypotheses:

1. Critical pH-induced structure-forming events will depend on the level of pea protein enrichment when mixed with GA;
2. Complexation of GA to both pea Lg and Vn protein isolates will result in a partial unfolding of their tertiary structures;
3. Canola isolate-polysaccharide complexes will form close to, or above the isoelectric point of the protein depending on the polysaccharide present;
4. Coacervation of canola isolate-polysaccharide mixtures will be primarily driven by electrostatic attractive forces, with secondary effects arising from hydrogen bonding; and

5. The solubility of mixed protein-polysaccharide systems will be improved relative to proteins alone.

### **1.3 Objectives**

Objectives of this research were as follows:

1. To evaluate the effect of protein enrichment and pH on complex formation involving fractions of pea protein (mixed, Lg and Vn isolates) and GA;
2. To evaluate complexation-induced structural modifications of pea Lg and Vn fractions under pH conditions where soluble and insoluble complexes are formed;
3. To study the effect of solvent pH and biopolymer mixing ratio on complex formation involving a canola protein isolate with alginate and  $\iota$ -carrageenan;
4. To study the nature of inter- and intra-molecular forces involved with complex formation in admixtures of canola protein isolate and alginate and  $\iota$ -carrageenan; and
5. To study the effect of pH on coacervate solubility of relative to homogeneous solutions of pea protein and canola isolates.

### **1.4. Terminology**

Within this thesis, the term complex coacervation refers to the associative process whereby biopolymers undergo a liquid-liquid phase separation to form coacervates or a liquid-solid phase separation to form precipitates (depending upon the biopolymer and solvent conditions). Mechanisms driving either coacervate or precipitate formation are considered to be similar, and as such the terms ‘complex’ or ‘complex formation’ are used interchangeably to describe this process. ‘Insoluble complexes’ is a term used to designate protein-polysaccharide complexes after significant liquid-liquid or liquid-solid phase separation occurred. This is not an indication of the complex’s functional properties (soluble vs. insoluble), which refers to the ability for the complex to remain suspended in solution. The term biopolymer refers to polymers of biological origin which include both plant proteins and polysaccharides.

## **CHAPTER 2.0 LITERATURE SURVEY**

### **2.1 Protein-polysaccharide interactions**

Protein-polysaccharide interactions have generated much attention over the past decades due to their structure-controlling role in foods and biomaterials (Doublier et al., 2000; Kaibara et al., 2000; Weinbreck et al., 2003a-b; Mekhloufi et al., 2005; Singh et al., 2007; Turgeon et al., 2007; Liu et al., 2009, 2010a-b), use in protein separation (Espinosa-Andrews et al., 2007), enzymatic immobilization (Kaibara et al., 2000), and in the design of delivery vehicles for bioactive compounds and pharmaceuticals (Zhu et al., 2007). Depending on the biopolymer characteristics (e.g., type, reactive groups, chain length, branching, flexibility, hydrophobicity, mixing ratio and concentration) and solvent conditions (e.g., pH and salts), segregative or associative liquid-liquid phase separation occurs (Schmitt et al., 1998; Turgeon et al., 2003; Weinbreck et al., 2003a; Choupra et al., 2006). Under dilute solvent conditions, mixtures are co-soluble and lack the significant interactions required to phase separate (Figure 2.1a; Ye, 2008). These systems remain thermodynamically stable, as the entropic contribution of free energy dominates over the enthalpic component (de Kruif and Tuinier, 2001). As the total polymer content is raised above a critical value, localized fluctuations in concentration become more pronounced, triggering thermodynamic instability (de Kruif and Tuinier, 2001; Turgeon et al., 2003). In general, mixtures become incompatible when biopolymers carry a similar net charge, separating into protein- and polysaccharide-rich phases (segregative behaviour), whereas those with an opposite net charge lead to reversible associative behaviour to give both solvent-rich and protein-polysaccharide complex-rich phases (associative behaviour; Figure 2.1c; Doublier et al., 2000).

### **2.2 Segregative phase separation**

Segregative phase separation arises in protein-polysaccharide mixtures when the system becomes thermodynamically incompatible, favouring biopolymer-solvent

interactions (i.e., protein-solvent or polysaccharide-solvent) over protein-polysaccharide interactions (Doublier et al., 2000; Turgeon et al., 2003). In other words, biopolymers tend to surround themselves with similar molecules (Gilsenan et al., 2003b). These conditions may arise when the protein interacts with neutral polysaccharides or the polysaccharide carries a similar net charge to the protein (Doublier et al., 2000; Fang et al., 2006). The latter is strongly dependent upon solvent pH relative to the pI of the protein, and the ionic strength of the solution. Segregative interactions can lead to two different solution behaviours depending on the total biopolymer concentration, under dilute biopolymer conditions, proteins and polysaccharides remain co-soluble in solution since there is an insufficient amount of biopolymers to phase separate. Under these conditions, the following may occur: a) conformational transformations of one biopolymer in the presence of a second (e.g., changing from an expanded coil conformation to a more compacted or ordered structure); b) changes to the rate/extent of conformational ordering (Glisenan et al., 2003a). Under more concentrated total biopolymer conditions, there is sufficient amount of interactions to lead to bulk phase separation into protein-solvent and polysaccharide-solvent phases (Figure 2.1b).

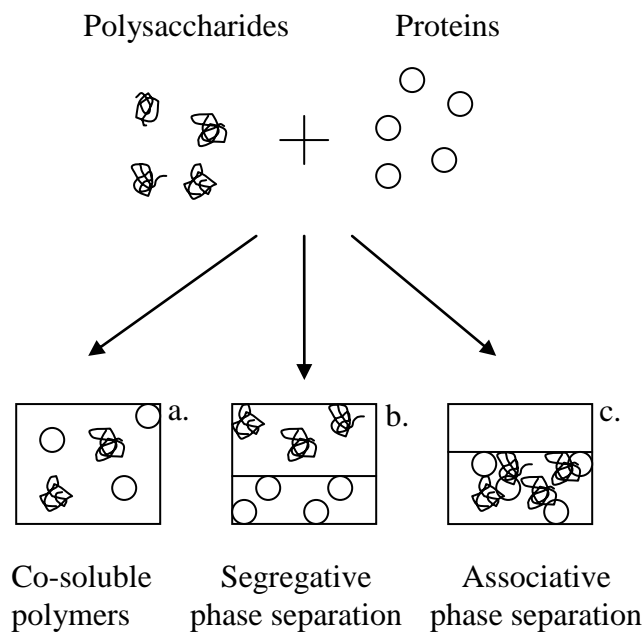


Figure 2.1. Segregative and associative phase behaviour in mixed protein-polysaccharide systems (adapted from deKruif and Tuinier, 2001).

### 2.3 Associative phase separation

Associative phase separation arises in protein-polysaccharide mixtures when the system becomes thermodynamically incompatible, whereby protein-polysaccharide interactions are favoured over biopolymer-solvent (i.e., protein-solvent and polysaccharide-solvent) (Doublier et al., 2000; Turgeon et al., 2003). This incompatibility leads to liquid-liquid (resulting in coacervates) or liquid-solid (resulting in precipitates) phase separation depending on the biopolymers involved and the solvent conditions. In either case, a biopolymer-rich and biopolymer-deficient phase is formed (Figure 2.1c). Conditions favouring associative phase separation arise when both biopolymers carry opposing net charges, and complex via both electrostatic attractive forces and non-covalent interactions (i.e. hydrogen bonding). Although the mechanism(s) governing associative phase separation have not yet been fully elucidated, the formation of protein-polysaccharide complexes is believed to follow a two-step nucleation and growth-type kinetic process associated with the formation of soluble and insoluble complexes (Weinbreck et al., 2003a; Girard et al., 2004). Complexation is typically followed by optical density analysis during a pH titration, with the formation of soluble complexes occurring at a pH associated with the first experimentally detectable non-covalent interaction between proteins and polysaccharides, as evident by an inflection point in the pH-optical density profile (denoted as  $\text{pH}_c$ ). The formation of insoluble complexes follows at  $\text{pH}_{\phi 1}$ , as evident by a rapid rise in optical density, and the transition from a transparent to cloudy sol (deKruif and Tuinier, 2001; Turgeon et al., 2003; Weinbreck et al., 2003a). Depending on the strength of interacting reactive groups along the polysaccharide backbone, precipitation may ensue or the formation of a coacervate structure with entrapped solvent may occur (Weinbreck et al., 2003a; Liu et al., 2009). In the latter case, insoluble complexes continually grow in size and number until reaching  $\text{pH}_{\text{opt}}$  (maximum coacervate yield), followed by their dissolution at  $\text{pH}_{\phi 2}$  when one of the biopolymers becomes protonated and no longer is electrostatically attracted to the other. Interactions are considered greatest when charges on opposing biopolymers become equivalent, leading to an overall neutral charge on the macromolecular complex (Ye, 2008). This tends to correspond to the maximum optical density found during the pH-titration and the highest coacervate yield, and is designated

as  $pH_{opt}$  (Liu et al., 2009). A stable coacervate-rich phase forms as entropies associated with biopolymer flexibility (or conformational entropy) and solvent mixing become reduced, which offsets the enthalpic contributions associated with the release of water and counter ions during complex formation (Schmitt et al., 1999; Singh et al., 2007; Ye, 2008). Figure 2.2 depicts a typical phase separation occurring during complex formation, where biopolymers of opposing charges first interact, and then phase separate into a coacervate-rich phase.

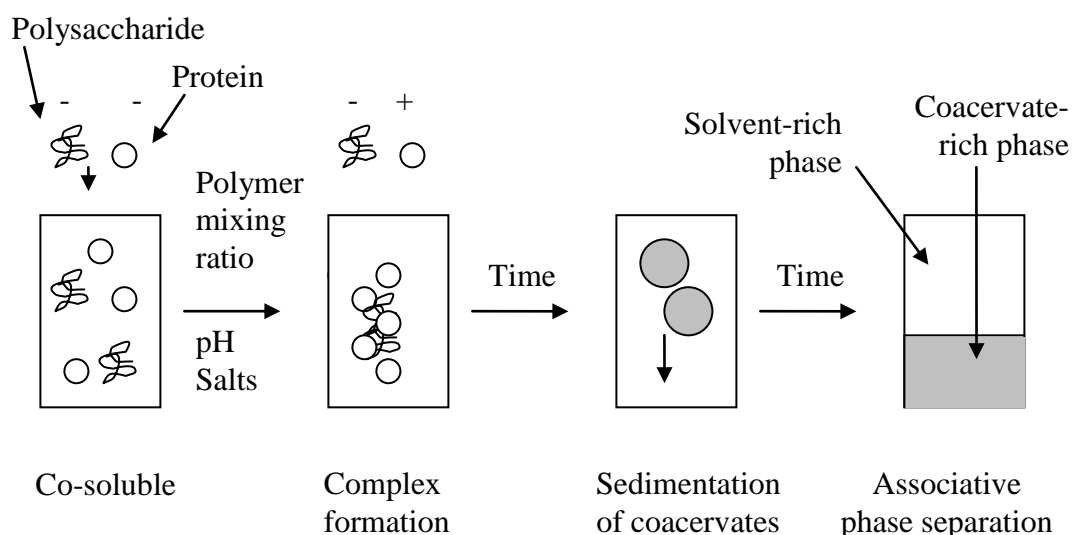


Figure 2.2. Formation of insoluble complexes that over time separate into a two phase system (complex coacervation) (adapted from Weinbreck, 2004).

Over the past few decades several theories have been developed to help explain the coacervation phenomenon. The theory of Overbeek and Voorn (1957) describes associative phase separation as a reversible spontaneous process when the charge density ( $\sigma$ ) and molar mass ( $r$ ) of the biopolymers involved are above the critical condition of  $\sigma^3 r \geq 0.53$ . The coacervate structure is formed via electrostatic attractive forces and remains dispersed by the entropy of mixing. Alternatively, Veis and Aranyi (1960) proposed the process to be a non-spontaneous two-step process whereby electrostatic attraction drives the formation of aggregates, which then rearrange to form



the coacervate phase. According to Veis and Aranyi, initial biopolymer interactions occur at  $\sigma^3 r < 0.53$ , with the majority of coacervate formation occurring when  $\sigma^3 r \geq 0.53$ . Tainaka (1979, 1980) extended the Veis-Aranyi theory to include the influence of charge density and biopolymer molar mass range of the biopolymers involved. Depending on these parameters, coacervates or precipitates would form, or biopolymers would remain co-soluble (both biopolymers remain in the same phase).

## 2.4 Nature of intra- and intermolecular interactions

Due to difficulties delineating contributions from each intermolecular force (electrostatic, hydrogen bonding and hydrophobic interactions), manipulation of the solvent environment (e.g., pH, urea, salts and temperature) is typically used to assess which forces dominate. Complex coacervation is generally reversible upon disruption of biopolymer electrostatic attractive forces through the addition of a high concentration of salt and/or by adjusting solvent pH so that biopolymers carry a similar net charge (Ye, 2008). Salts have the ability to disrupt the magnitude of electrostatic attractive forces between proteins and the polysaccharides. For example, the addition of  $\text{Na}^+$  and  $\text{Cl}^-$  ions screen the charges on the biopolymers, inhibiting coacervation. Changes in coacervate formation will be reflected by shifts in critical transitions within the phase diagram. Liu et al. (2009) found that at concentrations greater than 7.5 mM NaCl there was a formation of protein-protein aggregates at higher pH values than the onset of complex formation. This effect resulted in changes in the optical density profiles that interfered with reliable detection of critical pH values associated with complex formation. Liu et al. (2010a) also reported that at levels of 100 mM NaCl, coacervation in a pea protein isolate-GA system was prevented, indicating the primarily role of electrostatic attractive forces in complex formation. Similarly, Weinbreck et al. (2004a) reported a partial inhibition in complex formation for a whey protein isolate-carrageenan mixture at greater than 45 mM NaCl, with complete inhibition at 1 M NaCl. Electrostatic forces can also be disrupted when both biopolymers assume a net negative charge however, complexes may still form based on the strength of charged reactive groups on the polysaccharide. This is due to the electrostatic attraction of the proteins ( $-\text{N}^+\text{R}_3$ ) to the polysaccharides ( $-\text{OSO}_3^-$  or  $\text{COO}^-$ ).

The addition of urea to the solvent functions to disrupts hydrogen bonding allowing conclusions to be drawn regarding their role in coacervate formation and stability (Uruakpa and Arntfield, 2005). Some examples of the importance of hydrogen bonding in mixed systems are: a) Girard et al. (2002) reported that the addition of urea to  $\beta$ -lactoglobulin and low- and high- methoxy pectin systems led to a shifting in  $pH_c$  values indicating a secondary effect of hydrogen bonding on the formation of these complexes; b) Antonov and Soshinsky (2000) and Lii et al. (2002) reported that hydrogen bonding played an important role in rubisco-pectin and xanthan-gelatin systems, respectively. Liu et al. (2010a) found a secondary stabilizing effect of hydrogen bonding in a pea protein isolate-GA system.

Conducting pH-titrations at elevated temperatures functions to both reduce the effect of hydrogen bonding and enhance hydrophobic interactions (Kaibara et al., 2000; Weinbreck et al., 2004a). In literature, the formation of complexes has been reported to be independent of temperature for whey protein isolate-carrageenan (Weinbreck et al., 2004a) and BSA-poly(dimethyldiallylammonium chloride) mixtures (Kaibara et al. 2000). Liu et al. (2010a) reported no changes in the critical pH values describing complex formation in a pea protein isolate-GA system when the temperature was raised from 22°C to 60°C; indicating that hydrophobic interactions did not play a role in complex formation. However, the authors did report improved complex stability under acidic conditions (pH less than 3) at elevated temperatures (60°C) which was presumed to be associated with stronger hydrophobic interactions between protein-protein aggregates.

## **2.5 Factors affecting complex formation**

Complex formation can be influenced by a variety of factors, such as solvent conditions (e.g., pH and salts), biopolymer characteristics (e.g., type, reactive groups, chain length, branching, flexibility, hydrophobicity, mixing ratio and concentration) and processing conditions (e.g., pressure, temperature and shearing).

### 2.5.1 Effect of pH

Solvent pH is a key factor governing complex formation because of the ability to produce electrostatic attractive or repulsive forces between biopolymers. Solvent pH in relation to the native pI of the protein will dictate whether a protein carries a net positive or negative charge. Similarly, it also influences the level of protonation/deprotonation of reactive sites along the polysaccharide backbone. Typically, complex formation occurs over a narrow pH range, occurring between the pI of the protein and the pKa of the polysaccharide reactive sites (Weinbreck et al., 2003a; Duce et al., 2004). When solution pH is close to the pI of the protein, the charge on the protein is minimal and tends to inhibit coacervate formation. Liu et al. (2009) found that in a mixture of pea protein isolate (pI = 5.60) and GA (pKa = 1.88), complexation occurred over the pH range of 2.5 to 4.2, whereas Weinbreck et al. (2003a) reported whey protein isolate-GA mixed systems to complex between pH 2.3 and 5.3. At pHs outside of this range, significant electrostatic attraction between biopolymers did not occur. Absorption of a polysaccharide to the protein's surface also results in a shift in the native pI of the protein to lower pHs, affecting its behaviour in solution (Dickinson, 1998; Syrbe et al., 1998; Ye, 2008). Complex formation can be described during a pH-optical density scan, where critical pHs associated with structure-forming events, such as the formation of soluble ( $pH_c$ ) and insoluble ( $pH_{\phi 1}$ ) complexes, maximum biopolymer interactions ( $pH_{opt}$ ) and the dissolution of complexes ( $pH_{\phi 2}$ ) are identified. For instance, Liu et al. (2009) identified for a pea protein isolate-GA mixture in the absence of added NaCl, critical pH values ( $pH_c$ ,  $pH_{\phi 1}$ ,  $pH_{opt}$  and  $pH_{\phi 2}$ ) of 4.2, 3.7, 3.5 and 2.5, respectively. Above pH 4.2 and below pH 2.5, both the pea protein isolate and GA molecules were considered co-soluble and were not interacting via electrostatic attractive forces.

### 2.5.2 Effect of polysaccharide

Polysaccharides used for complexation are general classified into weakly or strongly charged polyelectrolytes (de Kruif et al., 2004). In general, weak polyelectrolytes (i.e., those having a relative low linear charge density) such as GA, low/high methoxy pectin, xanthan gum, carboxy methyl cellulose and guar gum form the liquid coacervate structure described previously. In contrast, strong polyelectrolytes

(i.e., those having a relatively high linear charge density or highly charged reactive group) such as chitosan (amine),  $\iota$ - /  $\kappa$ - carrageenan (sulphate), exocellular polysaccharide B40 (phosphate) and sulphated dextran, form precipitates. Linear charge density is defined as the number of charges present on the biopolymer per unit length (Schmitt et al., 1998). Reactive sites may be anionic or cationic in nature, influencing the pH regime where complexation with proteins occurs, or may be neutral to induce segregative effects. Polysaccharide structure, being linear or branched, also affects the ability for complex structures to form. Highly branched polysaccharides may reduce the availability of reactive sites due to steric hindrance. As such, depending on the number and type of reactive sites present, polysaccharides can have a dramatic effect on protein-polysaccharide interactions and complex formation.

Complex formation involving proteins and weakly charged polysaccharides occur below the pI of the protein, where the protein and polysaccharide have opposing charges. As examples: Weinbreck et al. (2003a) reported the formation of soluble complexes for a whey protein isolate (pI = 5.2)-GA occurred close to the pI of the protein; Liu et al. (2009) identified the formation of soluble complexes at pH 4.2 for a pea protein isolate (pI = 5.6)-GA mixture; Arntfield and Cai (1998) reported complex formation at pH 6.2 for a canola protein isolate (pI 6.8-7.2)-alginate system; and Schmitt et al. (1999) found that  $\beta$ -lactoglobulin (pI = 4.8)-high/low methoxy pectin systems complexed at pH 4.4. In contrast, complex formation involving proteins and strongly charged polysaccharides have been found to form at or above the pI of the protein where the net charge on the protein and polysaccharide is similar, resulting in liquid-solid phase separation or precipitation. Weinbreck et al. (2004a) reported complex formation for a whey protein isolate (pI = 5.2)-carrageenan mixture was initiated at pH 5.5, that was due to either electrostatic attraction forces arising with positively charged patches on the protein's surface, or via surface hydrophobic interactions. This type of localized attraction acts to overcome net repulsion between the two biopolymers to allow coacervation to occur (Schmitt et al., 1998). Dickinson (1998) and Doublier et al. (2000) reported that  $-\text{OSO}_3^-$  groups have greater attraction to  $-\text{N}^+\text{R}_3$  groups on the protein's surface than  $-\text{COO}^-$  groups.

### 2.5.3 Effect of total biopolymer concentration and mixing ratio

Typically in the literature, coacervation studies are carried out under dilute total biopolymer conditions (0.05-0.1% w/w), because at higher concentrations coacervation is suppressed. The latter is thought to be caused by: a) thermodynamic incompatibility of the mixture as biopolymers compete for available solvent, or b) an increased release of counter ions into solution screen reactive sites along the biopolymer's surface to reduce electrostatic attractive forces (Weinbreck et al., 2003b; Ye, 2008, Liu et al., 2009). Weinbreck et al. (2003a) reported for a whey protein isolate-GA mixture, that as the total biopolymer concentration (0.05%-1%) was increased,  $\text{pH}_{\phi 1}$  shifted to higher values, until an upper concentration limit was reached.

The biopolymer mixing ratio also plays a key role in complex formation, as this ratio influences the magnitude of the electrostatic attraction between the two biopolymers. As previously mentioned, the coacervation process is optimized (giving the highest yield) at biopolymer levels where stoichiometric (or electrical) neutrality occurs. If there is not enough protein in the system to neutralize charges on the polysaccharide then complexes will have a net negative charge. Schmitt et al. (1999) found that coacervates formed at all biopolymer mixing ratios (50:1-1:20 protein:polysaccharide) for a  $\beta$ -lactoglobulin-acacia gum system, when the total biopolymer concentration was greater than 1.0% (w/w) whereas for concentrations below this value coacervation only occurred when the protein was in excess. However, depending on the ratio, formed complexes still carried a net negative charge, as insufficient protein was present to neutralize all of the charges on the polysaccharide.

Figure 2.3 shows the relationship between pH and biopolymer mixing ratio on the formation of soluble ( $\text{pH}_c$ ) and insoluble ( $\text{pH}_{\phi 1}$ ) complexes, using a whey protein isolate-carrageenan system (Weinbreck et al., 2004a). The general trend finds soluble complex formation (at  $\text{pH}_c$ ) to be independent of the biopolymer mixture ratio. As examples, this trend was found for whey protein isolate-exocellular polysaccharide B40 (Weinbreck et al., 2003b), whey protein isolate-carrageenan (Weinbreck et al., 2004a) and  $\beta$ -lactoglobulin-acacia gum (Schmitt et al., 1999) systems. It was attributed to the interactions between individual proteins with individual polysaccharide chains. However, in all of the aforementioned work researchers used 'aggregate-free' protein

solutions. Alternatively, Liu et al. (2009) found a biopolymer mixing ratio dependence for  $pH_c$  when working with an aggregating pea protein isolate-GA system, whereby  $pH_c$  shifted to higher pH up to a protein-GA ratio of 4:1, before becoming constant at higher biopolymer mixing ratios. The authors attributed this dependence to the interaction between protein-protein aggregates with individual GA molecules, rather than individual protein molecules. Singh et al. (2007) also reported a similar biopolymer mixing ratio dependence for  $pH_c$  in type-A and type-B gelatin with agar, where  $pH_c$  increased up to a gelatin:agar ratio of 2, before becoming independent at higher values.

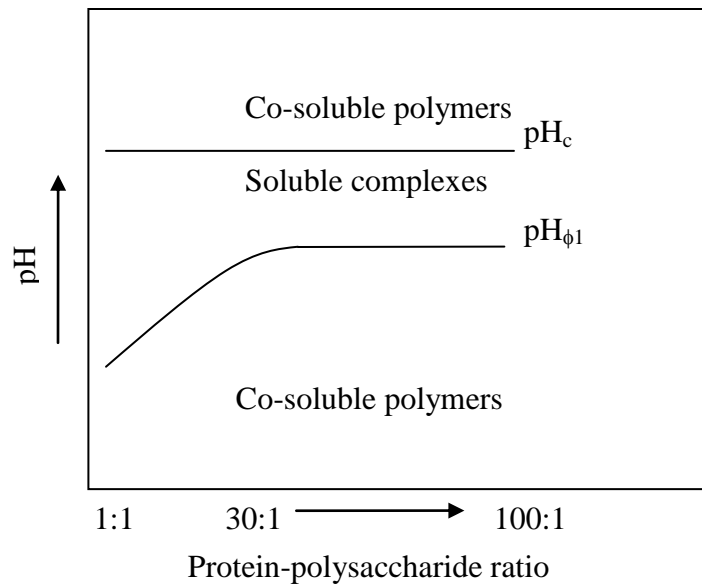


Figure 2.3. The effect of pH and biopolymer mixing ratio on critical pH values for a whey protein isolate-carrageenan mixture (adapted from Weinbreck et al., 2004a).

In contrast,  $pH_{\phi 1}$  has been found to be more sensitive to changes in biopolymer mixing ratio. For instance, Weinbreck et al. (2003b) found that in a whey protein isolate-exocellular polysaccharide B40 system,  $pH_{\phi 1}$  shifted to higher pH values as the biopolymer mixing ratios increased from 1:1 to 9:1. Above the 9:1 ratio,  $pH_{\phi 1}$  became constant as charges along the protein surface became saturated. Weinbreck et al. (2004a)

reported a similar trend for whey protein isolate-carrageenan mixtures, where  $\text{pH}_{\phi 1}$  increased up to a mixing ratio of 30:1 before levelling off. Similarly, Liu et al. (2009) reported that  $\text{pH}_{\phi 1}$  shifted to higher pH values in a pea protein isolate-GA system up to a mixing ratio of 4:1 before reaching a plateau. The shift in  $\text{pH}_{\phi 1}$  to higher pH values signifies the greater amount of protein molecules available per polysaccharide chain (Weinbreck et al., 2003b; Liu et al., 2009).

#### **2.5.4 Effect of salts**

The effect of salts on complex formation has been well studied in literature for systems such as:  $\beta$ -lactoglobulin-pectin (high and low methoxy) (Girard et al., 2002), whey protein isolate-GA (Weinbreck et al., 2003a), and pea protein isolate-GA (Liu et al., 2009). Figure 2.4 depicts the effect of pH and salt on the phase separation of a whey protein isolate-GA system. In general, under high ionic strength conditions, bound and unbound ions act to screen charges along the biopolymer's surface (electric double layer) so as to interfere with the electrostatic attraction between biopolymers of opposing charges. Weinbreck et al. (2003a) found that complexation of whey protein isolate and GA was inhibited at levels greater than 50 mM NaCl due to this charge screening effect. Liu et al. (2010a) reported similar results for pea protein isolate-GA mixtures at 100 mM NaCl. Girard et al. (2002) also found a significant reduction in complexation between  $\beta$ -lactoglobulin and high and low methoxy pectin at 110 mM NaCl. Under low ionic strength conditions, biopolymer-ion interactions may lead to a conformational change in the protein structure to expose buried or partially buried reactive sites (Weinbreck et al., 2003a; Duce et al., 2004). This phenomenon may lead to enhanced complexation between biopolymers. Weinbreck and co-workers (2004a) studied whey protein-carrageenan mixtures to find a shift in  $\text{pH}_{\phi 1}$  values toward higher pHs as NaCl levels increased up to 45 mM. The trend was also accompanied by a rise in maximum optical density as salt levels increased. At levels of 1 M NaCl, complexation of the whey protein-carrageenan mixture was prevented. Weinbreck et al. (2003b) reported that for a whey proteins and an exocellular polysaccharide EPS B40 mixture, both soluble and insoluble complexes formed at NaCl levels less than 75mM, however at levels greater than 75mM no phase separation was observed.

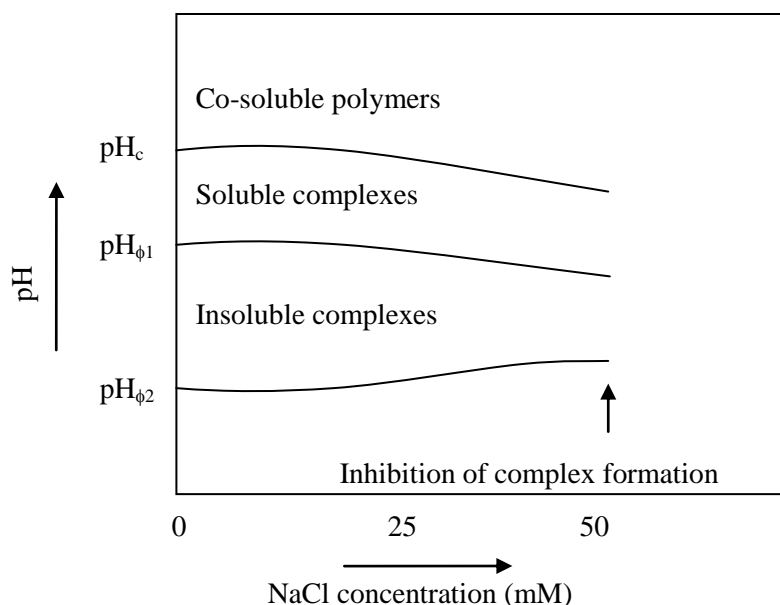


Figure 2.4. A typical phase diagram illustrating the effect of pH and ionic strength on critical pH values for a whey protein isolate-GA protein mixture (adapted from Weinbreck et al., 2003a).

Because the majority of studies in the literature have focused on the use of animal proteins, the effect of salts on complexation involving plant proteins is more limited. Liu et al. (2009) suggested that the presence of protein-protein aggregates played an important role in complex formation and stability within pea protein isolate-GA systems. The authors found that in the presence of low levels of salt, protein aggregation was promoted, so that soluble complexes formed as small aggregated clusters.

### 2.5.5 Effect of molecular weight

The molecular weight of the biopolymers within the mixed system also influences the coacervation process. Overbeek and Voorn (1957) predicted that an increase in biopolymer molecular weight would lead to an increase in degree of coacervation. From light scattering studies, Semenova (1996) showed that as the



molecular weight of dextran molecules increased so too did the level of coacervation with soy globulin. The authors presumed this effect to be associated with an increase in the volume occupied by the polysaccharide in solution, enabling greater access to the globulin proteins. Shieh and Glatz (1994) reported that biopolymer molecular weight played only a minor role in complex formation within lysozyme-polyacrylic acid mixtures, but it did have an effect on increasing the yield of precipitates formed in solution.

### **2.5.6 Effect of processing conditions**

Physical and mechanical factors, such as temperature, shearing and pressure have been shown to effect complex formation, and the stability of formed complexes. Temperature can influence the coacervate stability and/or coacervate formation by favouring hydrophobic interactions at higher temperatures; or at lower temperatures where hydrogen bonding is favoured (Schmitt et al., 1998). Raising the temperature influences protein conformation as partial or complete denaturation may occur, exposing buried reactive sites. This change may induce protein-protein aggregation, effectively increasing the molecular weight of the interacting species. Studies relating shearing rates (the rate at which force is applied to an object leading to deformation) and times to coacervate formation have been limited, but are thought to influence the number, size and shape of formed complexes in solution (Schmitt et al., 1998). Similarly the effect of pressure on coacervate formation is less understood, but is believed to influence protein conformation (partial denaturation) which in turn influences protein-polysaccharide interactions.

## **2.6 Role of protein aggregation in complex formation**

Formation of protein-protein aggregates during a pH-titration will influence the coacervation process by: a) creating larger complexes (increase in molecular weight) for interactions with polysaccharides; b) restricting the number of reactive sites available on the protein's surface for complexation; and c) altering the biopolymer mixing ratio required to reach neutrality and maximum yield. Schmitt et al. (2001) found that for a  $\beta$ -lactoglobulin-acacia gum system, ~60-70% of the acacia gum reacted with the

aggregates and formed precipitates. Liu et al. (2009, 2010a) reported in a pea protein isolate-GA system that aggregation played a key role during the formation of soluble complexes and in the stability of complexes at low solution pH (less than 3) due to stabilizing hydrophobic interactions within the aggregated structure. Aggregates have also been shown to play stabilizing role in  $\beta$ -lactoglobulin-acacia gum systems, relative to systems without aggregates (Schmitt et al., 2000; Sanchez and Renard, 2002). Schmitt et al. (2000) reported that  $\beta$ -lactoglobulin-acacia gum systems which contained aggregates were more stable based on the protein aggregates size distribution, and hypothesized that this was due to non-Coulombic interactions. In contrast, aggregate-free solutions form complexes via electrostatic attraction only, and have limited stabilizing effects of hydrophobic interactions. Weinbreck et al. (2003a) studied an aggregate-free system for whey protein isolate-GA where GA chains interacted with only a few protein molecules to form complexes. A better understanding of the role protein aggregates could play in the coacervation process may lead to formulated protein-polysaccharide ingredients that remain stable without the need for covalent crosslinking.

## **2.7 Modification to protein conformation upon complex formation**

Studies regarding protein conformational stability during complex coacervation have been limited, despite its potential for providing significant insight into the structure-function mechanisms of the process. Turgeon et al. (2003) used circular dichroism to describe changes in secondary structures upon complexation of  $\beta$ -lactoglobulin-acacia gum, and faba bean legumin-chitosan complexes. The authors suggested that the amount of protein-polysaccharide interaction increased as the protein unravelled.

Chourpa et al. (2006) used Raman microspectroscopy to study conformational changes in pea globulin and wheat gliadin under varying pH conditions. They found that for gliadin, the  $\beta$ -sheet fraction increased up to a pH of 3.0 and was greater than the random coil conformation over the pH range studied. In contrast, pea globulin was dominated by random coils at acidic pH. In gliadin, both random coils and  $\beta$ -sheets are created from  $\alpha$ -helices at acidic pH values. When GA is added to the system containing

pea globulin at pH 2.75 both  $\alpha$ -helices and  $\beta$ -sheets increased with a decrease in random coils. At a pH of  $\sim 4.0$  random coils and  $\alpha$ -helices were favoured for both proteins. These results indicate that the optimum pH for complex formation was 2.75 for globulin and 3.0 for gliadin as this is where the interactions between the protein and polysaccharide are the strongest (Choupra et al., 2006).

Schmitt et al. (2001) found that for complex formation between  $\beta$ -lactoglobulin and acacia gum the mechanism involved  $\alpha$ -helices located closer to the protein surface and upon complexation there was a decrease in  $\alpha$ -helices measured. These regions have a high charge density which leads to the electrostatic attraction. The  $\beta$ -sheets and aromatic residues were not affected by the electrostatic interactions. This information provides knowledge that is invaluable when tailoring protein-polysaccharide for specific approaches (Schmitt et al., 2001).

### **2.7.1 Fluorometry: as a means to study complexation-induced changes to protein structure**

Fluorometry or fluorescence spectroscopy is a technique used to assess the average surface hydrophobicity of a protein and/or conformational changes of the protein in response to processing or solvent conditions that cause partial or complete unfolding of the tertiary structure (Gorinstein et al., 2001; Lakowicz, 2006). The latter is reflected by increased fluorescence as buried aromatic amino acids (e.g, tryptophan, tyrosine and phenylalanine) become more exposed. The technique involves detecting fluorescence in which chromophores (e.g., exposed aromatic amino acids) absorb electromagnetic radiation to become excited to a higher vibrational energy state (internal conversion), followed by emission which occurs at a lower energy (longer wavelength) (Lakowicz, 2006). Data is reported as an 'intrinsic fluorescence' to give an indication of the solvent accessibility to the aromatic amino acids and the dynamics of the protein conformation. The high sensitivity of tryptophan makes it valuable for intrinsic protein fluorescence determination. This is why for proteins an excitation wavelength of 280 nm is often used, at this wavelength both tryptophan and tyrosine are excited. In most experiments phenylalanine is not excited this is due to its low quantum yield in proteins. Typically, the excitation wavelength is kept constant and fluorescence is measured as a

function of emission wavelength between 300 to 400 nm. Emission maxima for tryptophan, tyrosine and phenylalanine are seen at wavelengths of 348 nm, 303 nm, and 282 nm, respectively. Based on the shape of the fluorescence profile (i.e., peak height or fluorescence intensity (FI)) conclusions can be made on the protein structure (Lakowicz, 2006).

Aluko and Yada (1997) studied cowpea (*Vigna unguiculata*) and found that FI provided a good measure of protein surface hydrophobicity as a function of pH and NaCl. Gorinstein et al. (2001) measured the content of tyrosine, tryptophan and phenylalanine of amaranth and quinoa globulins by fluorometry. Amaranth globulins samples were shown to have high levels of phenylalanine and tyrosine when compared to quinoa. They also looked at the effect of urea on amaranth globulins. Based on shifting of the peaks they determined maximum denaturation occurred upon the addition of 8 M urea. Gorinstein et al. (2001) studied two varieties of amaranth and hypothesized that differences in denaturation may be due to the amino acids (denaturation will expose hydrophobic amino acids) and the disulphide bonds present in the protein.

## **2.8 Solubility**

Protein solubility is an important functional attribute to study, because its ability to stay in solution influences other properties such as gelation, emulsification, and foaming (Tolstoguzov, 1991). Protein solubility is strongly influenced by the surface properties of the protein, such as the balance of hydrophobic and hydrophilic amino acid residues exposed to the aqueous solvent. Solubility tends to increase as protein-solvent interactions are favoured, and decreases as protein-protein interactions are favoured. Proteins containing high surface charge and low surface hydrophobicity are in general more soluble than ones with the opposite protein properties. Proteins in general are least soluble at their pI, where there is no net protein charge this means that as the pH value moves away from the pI (either higher or lower) the solubility of the protein will increase. The interactions of a protein and polysaccharide may have several different effects on the protein's solubility. The electrostatic attraction between the charged polymers may act to decrease protein solubility as its ability to interact with water may be reduced. However, conformational changes may also lead to the exposure or

concealment of hydrophobic residues, which leads to decreased or increased protein solubility, respectively. Protein solubility was reported to be increased upon complexation for soy protein isolate-xanthan gum systems (Xie and Hettiarachchy, 1997) and for pea legumin-chitosan systems (Braudo et al., 2001). However, Liu et al. (2010b) reported a broadening of the minimal solubility portion of the solubility curve upon the addition of GA to a pea protein isolate solution, and a shift in the solubility minimum relative to a pea protein isolate solution.

## **2.9 Choice of materials**

### **2.9.1 Proteins**

The present research focuses on polysaccharide interactions with both pea and canola protein isolates. Pea proteins are commonly used as components of animal feeds and as plant protein ingredients (Koyoro and Powers, 1987). High protein crops are often processed into flour, protein concentrates and protein isolates (Sumner et al., 1981). Field peas (*Pisum sativum*) are dominated by two major globulin proteins: legumin (11S), and vicilin (7S) (Schroeder, 1982). Legumin is a hexamer (350-400 kDa) with six sub-units (~60 kDa) each comprised of  $\alpha$ - and  $\beta$ -chains held together by hydrogen bonding, van der Waals attractive forces and disulphide bridges (Ducel et al., 2004). Legumin is composed of 41%  $\beta$ -sheets, 16%  $\alpha$ -helices and has a small percentage of  $\beta$ -turns (Subirade et al., 1994). Vicilin is a trimer (150 kDa) comprised of three sub-units each 50 kDa in size. In contrast to Lg, Vn lacks disulphide bridging. Depending on the variety, the legumin:vicilin ratio can range between 0.2-1.5 (O’Kane et al., 2004). Convicilin is a minor storage protein present in pea seeds, it has a molecular weight of 71 kDa, and contains sulphur amino acids. Pea proteins exhibit similar functionality as soy protein products. However, they have lower water retention and gelling properties (Lampart-Szczapa, 2001). Pea legumin is considered to be non-gelling, whereas the vicilin fraction can form gels. In contrast, both the 11S and 7S proteins in soy are gel-formers. In general, the emulsification and foaming properties of pea are similar to soy (Lampart-Szczapa, 2001).

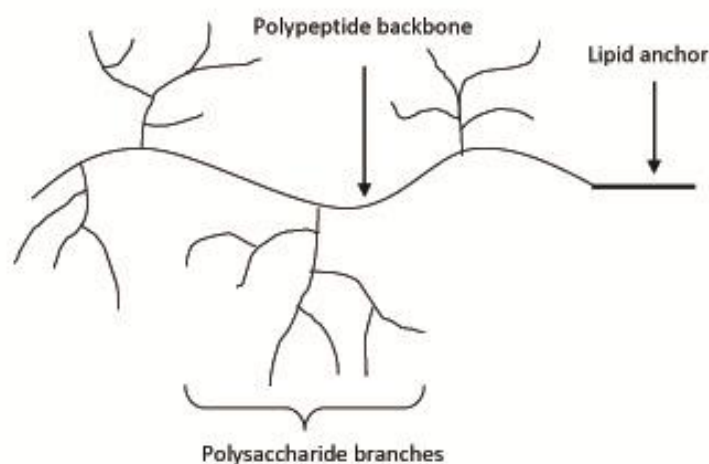
Canola is primarily grown for the production of canola oil, a high quality, healthy edible oil, which can also be used in the manufacturing of products such as

biodiesel and bioplastics (Wu and Muir, 2008). Canola meal is a by-product from oil production and is primarily used as an animal feed for livestock and poultry or as a fertilizer (Leger and Arntfield, 1993; Wu and Muir, 2008). The protein content in canola meal is approximately 40%, and has a balanced amino acid profile, being high in essential amino acids such as lysine (6%) and sulphur containing amino acids (3-4%) (Uruakpa and Arntfield, 2006; Wu and Muir, 2008). While canola meal has a high protein level other factors have limited its use as a food ingredient including high levels of fibre, polyphenols and phytic acid, along with inferior physiochemical properties and an undesirable colour and taste. Structurally, canola proteins are dominated by two main storage proteins: 12 S cruciferin and 2 S napin. Cruciferin (pI ~7.2) is a hexameric glycosylated (~12.9%) protein with a molecular weight of ~300 kDa; it has six subunits comprised of  $\alpha$ - (30 kDa) and  $\beta$ - (20 kDa) chains linked together by a total of 12 intramolecular disulphide bonds (Lampart-Szczapa, 2001). In contrast, napin (2S, pI 9-11) is a water-soluble albumin (12-14 kDa), containing two polypeptide chains of 4.5 kDa and 10 kDa held together by disulphide bonds (Berot et al., 2005). Canola protein isolates tend to be comprised of a mixed composition, with a wide variety of isoelectric points (pH 4-11) and molecular weights (13-320 kDa) leading to typically a low protein extraction yield when solvent extraction is used. Typically cruciferin- and napin-rich isolates can be produced by altering the extraction process of the meal. Cruciferin and napin represent ~60% and ~20% of the total seed protein, respectively. Functionally, canola proteins are considered to be gel formers (Lampart-Szczapa, 2001). Leger and Arntfield (1993) and Wu and Muir (2008) reported much better gelling properties for cruciferin (stronger gel networks) than napin (weaker gel networks). Canola proteins also have good lipid holding capacities, and emulsifying and foaming abilities (Lampart-Szczapa, 2001).

### **2.9.2 Polysaccharides**

The present research investigated complex formation involving pea proteins and GA, and canola proteins with alginate and  $\iota$ -carrageenan. The polysaccharides are widely used by the food industry either as thickeners or gelling agents, but also have surface active properties for use as emulsifiers or foaming agents (Dea, 1989). Gum

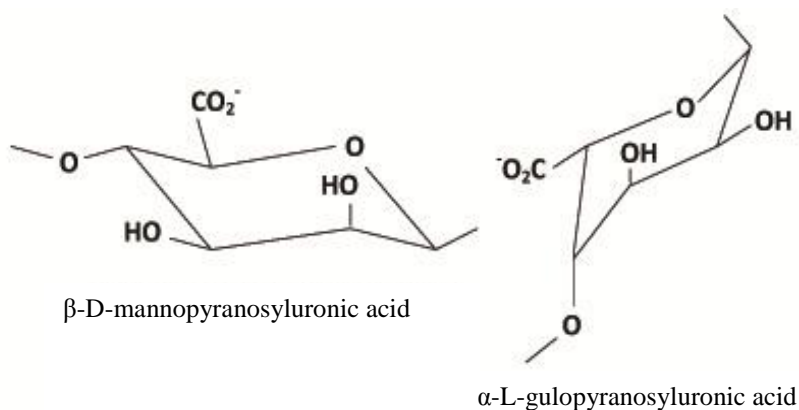
Arabic was chosen for complexation studies with the pea legumin and vicilin to build on the earlier work of Liu et al. (2009, 2010a-b) and to better understand the contribution of each protein fraction to complex formation observed for the mixed isolate. Gum Arabic is an anionic carboxylated polysaccharide extracted from *Acacia senegal* and *Acacia seyal* trees. Structurally, it is comprised of three fractions: a) a galactopyranose (galactan) polypeptide backbone with branched side chains of  $\beta$ -(1 $\rightarrow$ 6) galactopyranose (~89%) with terminating residues of arabinose, glucuronic acid and/or 4-O-methyl glucuronic acid; b) a covalently linked arabinogalactan-protein complex (~10%); and c) a glycoprotein complex (~1%) (Dror et al., 2006) (Figure 2.5).



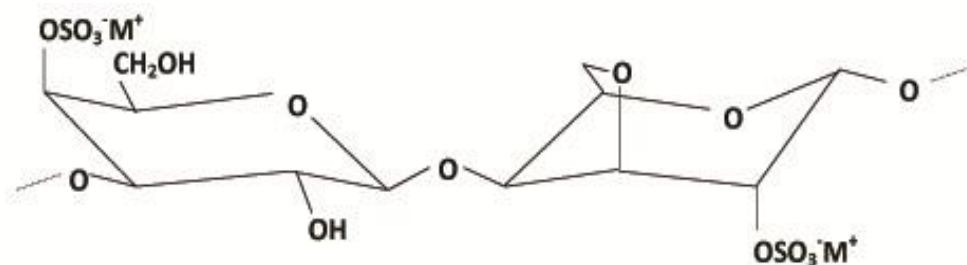
**Figure 2.5 Structure of gum Arabic (adapted from Islam et al., 1997).**

Alginate and  $\iota$ -carrageenan polysaccharides were chosen for complexation studies with canola protein isolate to compare the effect of differing reactive groups on complex formation. Alginate is a linear carboxylated polyuronic polysaccharide extracted from brown seaweed (*Phaeophyceae*), and consists of (1 $\rightarrow$ 4)-linked blocks of poly- $\beta$ -D-mannuronic acid (M), poly- $\alpha$ -L-guluronic acid (G) and mixed MG blocks (Harnsilawat et al., 2006) (Figure 2.6). In contrast,  $\iota$ -carrageenan is a linear sulphated polysaccharide extracted from red seaweed (*Rhodophyceae*) comprised of repeating disaccharide units of 1,3- $\beta$ -D- galactopyranose-4-sulphate and 1,4- $\alpha$ -D-3,6-anhydro-D-

galactose-2-sulphate, with each residue containing a sulphate group (te Nijenhuis, 1997; de Jong and van de Velde, 2007) (Figure 2.7). Both polysaccharides are also considered to be calcium sensitive, especially as it relates to their gel-forming abilities (te Nijenhuis, 1997; BeMiller and Huber, 2008). Although not the emphasis of this research, the effect of calcium complex formation, aggregation and structure-forming abilities on the macroscopic level would make for an interesting future study.



**Figure 2.6** Structure of alginate units (adapted from BeMiller and Huber, 2008).



**Figure 2.7** Structure of  $\kappa$ -carrageenan (adapted from de Jong and van de Velde, 2007).



## CHAPTER 3.0 MATERIALS AND METHODS

### 3.1 Materials

Canola seed (SP Desirable *Brassica napus*, Lot#: 168-8-129810, 2008), gum Arabic (GA) (FT Pre-hydrated, Lot#: 11229, 2007) and pea flour (PF) (Fiesta Flour, Lot#: F147X, 2008), were kindly donated by, Viterra (Saskatoon, SK), TIC Gums (Belcamp, MD) and Parrheim Foods Ltd. (Saskatoon, SK), respectively. Dipotassium hydrogen phosphate ( $K_2HPO_4$ ), glucono- $\delta$ -lactone (GDL), hexane,  $\iota$ -carrageenan (Type V, Batch # 064K1289), 2-mercaptoethanol, sodium alginate (Batch#: 035K0205), sodium azide ( $NaN_3$ ), sodium dodecyl sulphate (SDS), sodium phosphate ( $Na_2HPO_4$ ), urea were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). boric acid ( $H_3BO_3$ ), citric acid, ethyl alcohol, N-point indicator, pH buffers (2.00, 4.00 and 7.00), potassium dihydrogen phosphate ( $KH_2PO_4$ ), sodium chloride (NaCl), sodium hydroxide (NaOH) and Tris-base were obtained from VWR International (Mississauga, ON). Hydrochloric acid (HCl) and sulphuric acid ( $H_2SO_4$ ) were manufactured by EMD Chemicals Inc. (Gibbstown, NJ). Bio-Rad protein reagent and the BSA standard quick start kit were purchased from Bio-Rad (Mississauga, ON). All chemicals used in this study were of reagent grade.

The water used in this research is labelled Milli-Q<sup>TM</sup> water and was produced using the Millipore water system (Millipore Corporation, Milford, MA).

### 3.2 Chemical composition analysis

Chemical analyses for the protein-based materials (PF, ground canola seed, defatted canola meal, and canola protein isolate (CPI) were performed according to Association of Official Analytical Chemists (AOAC) Methods 925.10, 923.03, 920.87 and 920.85 for moisture, ash, crude protein and lipid (% wet weight basis), respectively (AOAC, 2003). Carbohydrate content was determined based on percent differential from 100%. Methods are summarized briefly below. A proximate analysis was not performed on the pea protein isolates due to limited amounts of material available for testing.

Instead, the Bradford dye-binding assay was used for determining their protein contents. In the case of GA, alginate and ι-carrageenan, only moisture, ash and carbohydrate content were taken into account for the proximate analysis, where protein and lipid levels were considered to be negligible.

### 3.2.1 Protein

The protein content for all materials was determined using the Kjeldahl method (except those stated above). In brief, 0.5 g of the dried materials were weighed into separate digestion flasks, followed by the addition of 25 mL of concentrated H<sub>2</sub>SO<sub>4</sub> and a catalyst (10 g K<sub>2</sub>SO<sub>4</sub>, 0.3 g CuSO<sub>4</sub>; Kjel-Pak Mixture # 200; VWR International, Mississauga, ON)). Flasks were then placed in a digestion block (Model K-435, Büchi Analytical Inc., Postfach , Switzerland) and digested for 1 h. After cooling, 70 mL of Milli-Q water was added to each digestion flask. Following digestion, flasks were distilled using a Büchi® steam distillation unit (Model B-324, Büchi Analytical Inc., Postfach, Switzerland) which automatically adds 100 mL of 30% (w/v) NaOH and 25 mL of 4% (w/v) boric acid. Approximately 100-150 mL of distillate was collected in a 250 mL Erlenmeyer flask containing 4 drops of N-point indicator (methanol, water, bromocresol green, methyl red). Samples were titrated with 0.2 M HCl until the solution colour turned light pink. Percent nitrogen (%N) was determined by:

$$\% \text{Nitrogen} = \frac{(\text{mL HCl for sample} - \text{mL HCl for blank}) * 0.2 \text{ M HCl} * 14.007 \text{ g/mol} * 100}{\text{Sample weight (mg)}} \quad (\text{eq.1})$$

Percent protein was determined by multiplying %N by 6.25 (nitrogen conversion factors) for all materials, except canola where a conversion factor of 5.70 (Uruakpa and Arntfield, 2006). All measurements were made in triplicate.

### 3.2.2 Moisture

Moisture content was determined gravimetrically using a forced air drying oven (Fisher Scientific, Ottawa, ON). Aluminium dishes (57 mm) were pre-dried for 16 h at

100-102°C, followed by cooling to room temperature (21-23°C) (~30 min) within a glass dessicator containing drierite dessicant (Fisher Scientific, Ottawa, ON). Meal and seed samples were ground using a coffee grinder for 20 s, and then weighed (2-4 g  $\pm$  0.0002 g) into the pre-dried aluminium dishes using an analytical balance (Metler, Columbus, OH). Samples were then dried at 100-102°C for 16-18 h. Samples were then placed in the dessicator to cool for 30 min prior to weighing. The percent moisture content was calculated using the following equation:

$$\% \text{ Moisture} = 1 - \frac{[(\text{weight of dried sample (g)} + \text{weight of dry dish (g)}) - \text{weight of dry dish (g)}]}{\text{Sample weight (g)}} * 100$$

(eq. 2)

All measurements were made in triplicate.

### 3.2.3 Lipid

A Goldfish Extractor (Labconco Corporation, Kansas City, MO) was used to determine the sample lipid content. Samples were weighed (2-4 g  $\pm$  0.0002 g) using the pre-dried samples from Section 3.2.2. Samples were transferred onto a 90 mm filter paper (Whatman No. 4), folded to prevent any loss of sample, and placed within a thimble (25 x 80 mm) (Ahlstrom AT, Holly Spring, PA). These were then placed into metal tubes and placed on the Goldfish extractor unit. Hexane (50 mL) was added, to pre-dried oil extraction beakers (dried at 100-102°C for 16 h) and placed on the extractor. Extraction was then performed for 6 h. Afterwards, hexane was collected, followed by an additional evaporation step to remove any remaining hexane from the extraction beakers using a hot plate (30-40°C) in a fume hood. The beakers containing the oil were oven dried (100-102°C) for 30 min, followed by cooling in a dessicator for 30 min and weighed. Lipid content (%) was determined using the following equation:

$$\% \text{ Lipid Content} = \frac{[(\text{weight of dried beaker} + \text{oil (g)}) - \text{weight of dry beaker (g)}]}{\text{Sample weight (g)}} * 100$$

(eq. 3)

All samples were analyzed in triplicate.

### 3.2.4 Ash

A muffle furnace (Fisher Scientific, Ottawa, ON) was used to determine sample ash content. Crucibles (Fisher Scientific, Ottawa, ON) were pre-dried at 100-102°C in a drying oven for 16 h, followed by cooling to room temperature (~30 min) within a glass dessicator. Samples were weighed (1-3 g  $\pm$  0.0002 g) using an analytical balance and placed directly into the pre-dried pre-weighed crucibles, followed by heating on a hot plate (70-80°C, 30 min) until the samples turned black to prevent ignition of the samples within the furnace. The crucibles were then transferred to the muffle furnace and heated at 500-505°C for 24 h until the samples became white in colour. Crucible were transferred to a dessicator for 1 h prior to final weighing. All samples were measured in triplicate. The ash content (%) was calculated using the following equation:

$$\% \text{ Ash} = \frac{[(\text{weight of ash (g)} + \text{weight of dry crucible (g)}) - \text{weight of dry crucible (g)}] * 100}{\text{Sample weight (g)}} \quad (\text{eq. 4})$$

### 3.2.5 pH determination

Sample pH was determined using an Accumet, Model 15 pH meter with a glass body combination electrode (Fisher Scientific, Ottawa, ON). A three point calibration was performed using pH 7.00, 4.00 and 2.00 buffers to standardize the pH meter. All pH values are reported  $\pm$ 0.002 units.

### 3.2.6 Conductivity

Sample conductivity was measured using an Orion 3 Star Conductivity Meter with a dura probe 4 electrode conductivity cell (Thermo Scientific, Edmonton, AB).

## 3.3 Coacervation of pea proteins and gum Arabic (Study 1)

### 3.3.1 Preparation of protein isolates

Separation of pea proteins from PF was performed by salt/pH extraction to yield crude isolates of Lg and Vn, and a mixed isolate based on modification of the method of

Bora et al. (1994). The crude Lg and Vn isolates were later enriched using a low pressure-liquid chromatography system (BioLogic DuoFlow, Bio-Rad, Mississauga, ON) with DEAE (LPLC-DEAE) (DEAE Sepharose fast flow, GE Healthcare Bio-Sciences Inc., Baie d'Urfé, PQ) as the stationary phase.

*(a) Preparation of crude pea protein isolates:*

To summarize, 250 g of PF was dissolved in 1.5 L of a pH 7.20  $\text{K}_2\text{HPO}_4$  buffer (50 mM) containing 0.5 M NaCl (volume-weight ratio of 6 mL buffer per g PF), and then stirred continuously using a magnetic stirrer for 1 h at room temperature (~21-22°C). The mixture was centrifuged at 18,600 x g for 15 min at 4°C (Beckman J2-HC, Beckman Coulter, Brea, CA). The supernatant was collected and filtered using glass wool to remove large undissolved particles. The filtered supernatant was then diluted using 5 volumes of cold (4°C) Milli-Q water, adjusted to pH 4.50 using 2 N HCl, and then left to stand overnight (16 h) within a cold room (4°C) to facilitate settling of the salt-soluble proteins. The majority of the supernatant was decanted off and the remaining precipitate and supernatant was collected by centrifugation. The recovered pellet was washed with 200 mL of Milli-Q water, followed by another centrifugation step. The washed pellet was re-suspended within the same buffer at a volume-weight ratio of 5 mL per g pellet, and then allowed to stir continuously for 1 h at 4°C. This last extraction step was repeated, and subsequent supernatants pooled. Approximately one third of the supernatant was adjusted to pH 4.50 using 1 M HCl with continuous stirring, followed by centrifugation (18,600 x g, 15 min, 4°C) to collect the protein-rich pellet. This pellet was subsequently washed with 50 mL of Milli-Q water and then re-centrifuged. The pellet was then suspended in Milli-Q water and placed into dialysis tubing (6-8 kDa exclusion limit; Spectrum Laboratories, Inc., Rancho Dominguez, CA). These tubes were then placed within 15-20 L of Milli-Q water and dialyzed, changing the Milli-Q water every hour only during the day until the conductivity of the dialysis water reached 1.5  $\mu\text{S}/\text{cm}$  (~24 h). The de-salted material was then freeze dried for 72 h using a Labconco freeze drier (model 7750000, Labconco Corporation, Kansas City, MO) and stored in plastic screw capped 50 mL tubes at -20°C. This fraction was labelled the 'mixed protein isolate' The remaining two thirds of the supernatant were

dialyzed against a McIlvaines buffer (0.2 M  $\text{Na}_2\text{HPO}_4$  and 0.1 M  $\text{C}_6\text{H}_8\text{O}_7$  at pH 4.8) containing 0.2 M of NaCl at 4°C for 42 h, with the buffer being changed twice (at 16 and 25 h) using a volume ratio of 10 mL buffer per 1 mL of supernatant. The Lg-rich precipitate was collected by centrifugation (18,600 x g, 15min, 4°C) and the Vn-rich supernatant was saved for later use. The pellet was washed, re-centrifuged and dialyzed as previously described. The de-salted material was then freeze dried (see above) and labelled as the 'crude Lg isolate'. The supernatant was then adjusted to pH 4.50 using 1.0 M HCl to precipitate the Vn proteins, followed by centrifugation, washing, re-centrifugation and dialysis as previously described. The de-salted crude fraction was then freeze dried and labelled as the 'crude Vn isolate'.

*(b) Preparation of enriched pea Lg and Vn isolates:*

Crude Lg and crude Vn isolates were submitted to LPLC-DEAE chromatography system. Crude protein isolates of Lg and Vn (10 mg-small column or 250 mg-large column) were individually dissolved in a pH 8.00  $\text{K}_2\text{HPO}_4$  buffer (35 mM) containing 0.075 M NaCl and 0.02% (w/v)  $\text{NaN}_3$ , and sonicated for 5 min to help dissolve the sample (Branson 2510, Branson Ultrasonics Corp., Danbury, CT), samples were added directly to individual columns. The small column (1.5 cm x 50 cm) with a DEAE resin height of 35 cm and a flow rate of 0.2 mL/min was used for estimating the Lg/Vn ratios and protein recovery. For scale up, a larger column (3 cm x 120 cm) with a DEAE resin height of 95 cm and a flow rate of 0.7 mL/min was used. Fractions were eluted in a step-wise manner using two buffers with different NaCl concentrations: buffer 1) pH 8.00  $\text{K}_2\text{HPO}_4$  (35 mM) containing 0.15 M NaCl and 0.02% (w/v)  $\text{NaN}_3$  to elute an enriched Vn isolate, followed by, buffer 2) pH 8.00  $\text{K}_2\text{HPO}_4$  (35 mM) containing 0.40 M NaCl and 0.02% (w/v)  $\text{NaN}_3$  at pH 8.00) buffer to elute an enriched Lg isolate. Peaks were detected using a UV lamp at 280 nm. Enriched isolates were dialyzed against 15-20 L of Milli-Q water (until the dialysis water conductivity reached 1.5  $\mu\text{S}/\text{cm}$ ), freeze-dried (see above), placed in 50 mL plastic screw capped tubes and stored at -20°C (Bora et al., 1994). During the enrichment process, it was assumed that equal Lg/Vn ratios were obtained using both columns.

*(c) Protein determination by the Bradford Assay:*

Protein content in the crude and enriched protein isolates and chromatographic fractions were determined using the Bradford protein assay (Bradford, 1976), due to the low quantities of materials obtained in section 3.3.1b. Two standard curves were prepared using known concentrations of BSA (0.04-0.90  $\mu\text{g}/\mu\text{L}$ ), using both Milli-Q water and the  $\text{K}_2\text{HPO}_4$  (35mM, 0.075M NaCl and 0.02% (w/v)  $\text{NaN}_3$  at pH 8.00) buffer. Freeze-dried crude and enriched protein isolates (~1 mg) were dissolved in 1 mL of Milli-Q water, sonicated for 5 min and vortexed. For the assay, 100  $\mu\text{L}$  of sample or standard was added to 5 mL of protein reagent (containing 4 mL Milli-Q water and 1 mL of the Bio-Rad dye reagent concentrate (Bio-Rad, Mississauga, ON)) in a glass test tube (1.8 cm x 15 cm) and mixed by inverting. Sample absorbance was determined at 595 nm using a Genesys 10 UV-visible spectrophotometer (Thermo Scientific, Waltham, MA) and plastic cuvettes (1 cm path length) after a 15 min incubation period at room temperature. Sample protein content was determined from the BSA standard curve with either Milli-Q water (isolates) or phosphate (chromatographic fractions). If required, sample solutions were diluted with extraction buffer to ensure that readings were within the range of the BSA standard curve. Crude and enriched isolates protein contents were performed in duplicate. Protein content from chromatographic fractions was performed in triplicate.

### **3.3.2 Gel electrophoresis**

Pea flour and protein isolates (mixed isolate, crude Lg, enriched Lg, crude Vn and enriched Vn) were characterized by both native and sodium-dodecyl sulphate (SDS) (non-reducing) polyacrylamide gel electrophoresis (PAGE) (PhastSystem, GE Healthcare Bio-Sciences Inc., Baie d'Urfé, PQ). Samples for native-PAGE were prepared by dissolving 3 mg of protein in 1 mL of 20 mM  $\text{K}_2\text{HPO}_4$  buffer (pH 7.50), followed by centrifugation (Eppendorf Canada, Mississauga, Ontario) at  $16,873 \times g$  for 10 min (Marcone et al., 1998). One microlitre of each protein solution was loaded onto a 4-15% gradient phastgel (pre-cast) and run using native phastgel buffer strips (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, PQ). Samples for non-reducing SDS-PAGE

were prepared by dissolving 2 mg of protein with 1 mL in a 0.1 M Tris-HCl buffer (containing 5% SDS (w/v) at pH 8.00), followed by heating at 99°C (Incu Block model 285, Denville Scientific Inc., South Plainfield, NJ) for 10 min to unravel and disassociate the protein, followed by cooling the solution to room temperature (21-23°C). Mixtures were centrifuged at 16,873 x *g* for 10 min to remove any insoluble material (Marcone et al., 1998). Protein solutions were run on 8-25% gradient phastgel using SDS phastgel buffer strips (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, PQ). Protein molecular weights were estimated using Image Master 1D Elite software (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, PQ) based on the following protein standards: 170, 130, 95, 72, 55, 43, 34, 26, 17 and 10 (kDa). Both native- and SDS-PAGE gels were stained using Coomassie Brilliant Blue dye (PhastGel Blue R, GE Healthcare Bio-Sciences Inc., Baie d'Urfé, PQ).

### **3.3.3 Optical density analysis**

Critical pH values associated with structure-forming events during complex coacervation were investigated by optical density analysis during an acid pH-titration for admixtures of GA with the various pea protein isolates (mixed isolate, crude Lg, enriched Lg, crude Vn and enriched Vn). Stock solutions of protein (0.066%, w/v at pH 8.00) and GA (0.033%, w/v at pH 8.00) were prepared by dissolving each powder in Milli-Q water, adjusting pH using 0.1 and 1.0 M NaOH and stirring for 2 h at room temperature, followed by mixing at 4°C for 16 h to allow prolonged exposure to solution at pH 8.00. Solutions were brought to room temperature before mixing of the stock solutions. Stock solutions were combined to obtain a volume of 40 mL at a 2:1 protein-GA mixing ratio and a total biopolymer concentration of 0.05% (w/v), and adjusted to pH 8.00 with 0.1 M NaOH. The ratio corresponds to the optimal biopolymer-mixing ratio for pea protein isolate and GA as determined by Liu and co-workers (2009). Acidification of the solution during optical density analysis was achieved through the addition of 0.04% (w/v) GDL (glucono delta-lactone) which brought the pH slowly down from 8.00 to 4.10, without significant dilution. Solution pH was lowered further using a gradient of HCl: 0.1 M HCl was used at solution pH greater than 3.50; 0.5 M was used in the pH range of 2.90 and 3.50; 1.0 M HCl was used in pH range of 2.40 and



2.90; and 2.0 M HCl was used at solution pH less than 2.40. Changing concentration of HCl allowed accurate changes of pH and a reduction in the total volume of HCl added. System conductivity was monitored starting at 136  $\mu\text{S}$  and ending at 6000-9000  $\mu\text{S}$ . However, during the complexation range (pH 5.00-2.40) the conductivity range was 155  $\mu\text{S}$  to 1300  $\mu\text{S}$ . A total volume of 1.2 to 1.4 mL of HCl was added to the solution with the majority (0.8-1.1 mL) being added below pH 2.50. Changes in optical density were measured as a function of pH (6.00-1.50) by spectroscopy at 600 nm using plastic cuvettes (1 cm path length). Critical pH values ( $\text{pH}_c$ ,  $\text{pH}_{\phi 1}$ , and  $\text{pH}_{\phi 2}$ ) were determined graphically as the intersection point of two curve tangents (Figure 3.1) (Weinbreck et al., 2003a; Liu et al., 2009). The pH where maximum optical density occurred was denoted as  $\text{pH}_{\text{opt}}$ . Pea protein isolates (0.033%, w/v) and GA (0.017%, w/v) solutions were measured as controls under the same titration conditions as in the mixed system. All optical density curves were performed in triplicate. Critical pH values were reported as the mean  $\pm$  one standard deviation of triplicate samples.

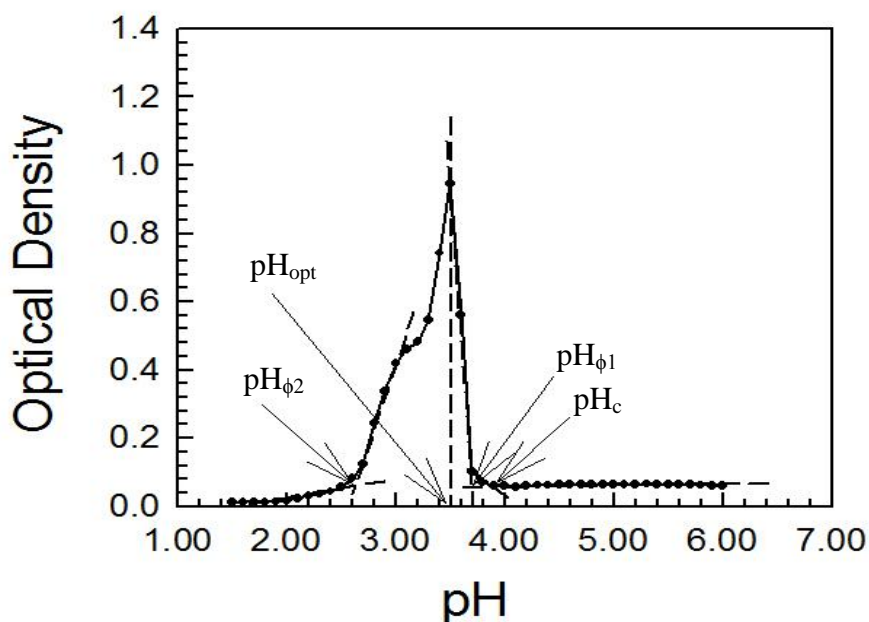


Figure 3.1. Mean optical density curve for crude Lg-GA system (biopolymer concentration of 0.05%, biopolymer ratio of 2:1 (w/v)). The critical pH transition points ( $\text{pH}_c$ ,  $\text{pH}_{\phi 1}$ ,  $\text{pH}_{\text{opt}}$  and  $\text{pH}_{\phi 2}$ ) are determined by tangent lines and indicate structure forming events.

### 3.3.4. Electrophoretic mobility (zeta potential)

Electrophoretic mobility ( $U_E$ ) (compound velocity in an electric field) for isolates and mixed protein isolates (crude Lg, enriched Lg, crude Vn and enriched Vn) and GA solutions were investigated as a function of pH (6.50-1.50) using a Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA). Samples were prepared in the same manner as section 3.3.3 for both the mixed and protein alone systems, except no GDL was used. Hydrochloric acid (0.1 M-2.0 M) was used to adjust each sample to pre-determined pHs. A separate sample (40 mL) was prepared for each pH value. By applying the Henry equation the electrophoretic mobility can be used to calculate the zeta potential ( $\zeta$ ), which gives an estimate of the surface charge on the biopolymer:

$$U_E = \frac{2\varepsilon \times \zeta \times f(\kappa\alpha)}{3\eta} \quad (\text{eq. 5})$$

Where,  $\eta$  is the dispersion viscosity,  $\varepsilon$  is the permittivity, and  $f(\kappa\alpha)$  is a function related to the ratio of particle radius ( $\alpha$ ) and the Debye length ( $\kappa$ ). Using the Smoluchowski approximation  $f(\kappa\alpha)$  equalled 1.5. All measurements were made in duplicate.

### 3.3.5 Fluorescence emission spectroscopy

Changes to the protein's tertiary conformation upon complexation was monitored as a function of pH and emission wavelengths (300-400 nm; at 0.5 nm increments) for only the enriched Lg and Vn isolates, with and without GA (at a 2:1 protein:GA mixing ratio, volume of 20 mL and a total biopolymer concentration of 0.05%, w/v). Fluorescence intensity (FI) was measured using the FluoroMax 4 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) at an excitation and emission wavelengths of 280 nm and 300-400 nm, respectively. Excitation and emission slit widths were set at 5 nm. All measurements were performed at room temperature, using a quartz cuvette (1 cm path length) and in duplicate (10 scans per sample). FI was monitored at pH values corresponding to conditions where soluble (4.00) and insoluble (3.70 for legumin and 3.60 for vicilin) complexes were present for the mixed systems.

Biopolymer solutions (Lg, Vn and GA) were also tested at similar pH values, plus 5.50 in which the biopolymers were considered to be co-soluble. FI data were reported as a function of emission wavelength, and the peak FI value was used.

### **3.3.6 Solubility**

Percent protein solubility was investigated as a function of pH (2.00-10.00) for admixtures of GA and pea protein isolates (i.e. mixed isolate, crude Lg and crude Vn) at a 2:1 protein-GA mixing ratio and a total biopolymer concentration of 0.1% (w/v). Samples were prepared as section 3.3.3 (using same volume and biopolymer mixing ratio), with adjustments to pH being achieved using either a HCl gradient (as described in Section 3.3.3) or NaOH (0.1 M or 1.0 M). After pH adjustment, samples were stirred for 1 h, and then centrifuged at 16,700 x g for 20 min at room temperature (Aluko and Yada, 1995). Protein content in supernatant was determined using Bradford assay and percent protein solubility was calculated by dividing the water-soluble protein content by the total protein content (x100%). Pea protein isolates were also tested at an equivalent concentration as found in the mixed system (0.067%, w/v). All measurements were performed in triplicate.

### **3.3.7 Statistics**

A one-way analysis of variance (ANOVA) with a Scheffe post-hoc test was used to measure statistical differences within state diagrams for each  $pH_c$ ,  $pH_{\phi 1}$ ,  $pH_{opt}$ , and  $pH_{\phi 2}$  between the mixed isolate-GA system and the crude and enriched Lg and Vn isolates. For fluorescence data, multiple comparison was used to test for pH differences in the mixed and protein alone systems. All statistical analyses were performed using Systat software (SPSS Inc., ver. 10, 2000, Chicago, IL).

## **3.4 Coacervation of canola protein isolate and alginate/ $\iota$ -carrageenan (Study 2)**

### **3.4.1 Preparation of canola protein isolates**

Defatted canola meal was prepared by pressing canola seed (~10 kg) using a continuous screw expeller (Komet Type CA59 C, IBG Monforts Oekotec GmbH & Co., Monchengladbach, DE), followed by hexane extraction at a 1:1 (w/v) meal to hexane

ratio for 16 h (adapted from Folawiyo and Apenten, 1996). The meal was placed in metal trays (36 cm x 48 cm) and air-dried for 8 h in a fume hood; hexane extraction was then repeated a second time. Proteins from the defatted meal were extracted using a Tris-base-HCl buffer (pH 7.00) containing 0.1 M NaCl at a ratio of 10 mL buffer/g meal for 2 h under constant mechanical stirring at room temperature. The dispersion was then centrifuged at 18,600 x g for 1 h at 4°C, and the supernatant was recovered. A second centrifuge step for 30 min was used to further clarify the supernatant of insoluble residues. The supernatant was dialyzed (6-8 kDa cut off) in 15-20 L of Milli-Q water at 4°C for 48 h with water changes every hour only during the day until the dialysis water reached a conductivity of 1.5 µS/cm. Precipitated proteins were recovered by centrifugation at 18,600 x g for 2 h at 4°C, and then subsequently freeze dried (see section 3.3.1a) to yield a canola protein isolate (CPI).

#### **3.4.2 Gel electrophoresis**

Reducing and non-reducing SDS-PAGE gels were run for the defatted canola meal and protein isolates described in Section 3.3.2, with the exception that samples were prepared at a concentration of 3 mg protein per mL buffer. In all cases, 8-25% gradient phastgels (pre-cast) using SDS phastgel buffer strips were used for the analysis. For reducing SDS-Page, 5% (v/v) 2-mercaptoethanol was added to the 0.1 M Tris-HCl buffer (containing 5% SDS (w/v) at pH 8.00) used to dissolve the protein. The same molecular weight standards and imaging analyzing software were used as described in Section 3.3.2.

#### **3.4.3 Optical density analysis**

*(a) Effect of biopolymer mixing ratio on the formation of soluble and insoluble CPI-polysaccharide complexes*

Critical pH values associated with the formation of soluble and insoluble complexes were investigated using optical density during an acid pH-titration for admixtures of CPI-alginate and CPI-ι-carrageenan at different biopolymer weight mixing ratios (CPI: polysaccharide mixing ratios of 1:1 5:1, 10:1, 15:1, 20:1, 25:1, 30:1 and 50:1). Canola protein isolate, alginate and ι-carrageenan samples were also analyzed

for comparative purposes. Biopolymer solutions (40 g) were prepared by dissolving each powder with Milli-Q water at the pre-determined mixing ratio to give a constant total biopolymer concentration of 0.05% (w/w). Solution pH was adjusted to pH 9.00 using 1.0 M NaOH, followed by mixing for 2 h at room temperature. Optical density measurements were performed in a similar manner as described in section 3.3.3 with optical density (at 600 nm) being read from pH 7.00 to 1.50. Solution pH was lowered using a gradient of HCl concentrations: 0.05 M HCl was used at solution pH greater than 6.00; 0.1 M HCl was used in the pH range of 3.50 and 6.00; 0.5 M HCl in the pH range of 3.00 and 3.50; 1.0 M HCl in the pH range of 3.00 and 2.50; and 2.0 M HCl at solution pH less than 1.50, no GDL was used. The amount of HCl required for each experiment was less than 2.50 mL with the majority of the volume (~2.00 mL) occurring below pH 2.5. Conductivity was monitored throughout the acid titration for three biopolymer mixing ratios (1:1, 20:1 and 50:1 CPI-polysaccharide). Systems having higher polysaccharide contents (i.e., 1:1 mixing ratio) had conductivities ranging from 39.7  $\mu\text{S}/\text{cm}$  at pH 7.00 to 41,700  $\mu\text{S}/\text{cm}$  at pH 1.50, whereas at ratios with higher levels of protein (i.e., 50:1) conductivity ranged from 56.4  $\mu\text{S}/\text{cm}$  at pH 7.00 to 44,400  $\mu\text{S}/\text{cm}$  at pH 1.50. Both the  $\iota$ -carrageenan and the alginate mixed systems showed similar increases in conductivity during the titration. Despite the large increases in conductivities at lower pH values, conductivities remained relative low (less than 200  $\mu\text{S}/\text{cm}$ ) at pHs corresponding to identified critical pH values ( $\text{pH}_c$  and  $\text{pH}_{\phi 1}$ ).

Canola protein isolate, alginate and  $\iota$ -carrageenan solutions were prepared as controls at equivalent concentrations found in the mixed systems. All optical density measurements were performed in triplicate.

#### *b) Nature of CPI-polysaccharide interactions*

The nature of biopolymer interactions were investigated by optical density analysis for a 20:1 CPI-polysaccharide mixing ratio only (total biopolymer concentration of 0.05%, w/w) during an acid titration (7.00-1.50) in the presence of destabilizing agents (0.1 M NaCl and 0.1 M urea) and at elevated temperatures (60°C). Changes to the pH-dependent optical density profiles (and associated  $\text{pH}_c$  and  $\text{pH}_{\phi 1}$

values) of CPI-alginate and CPI- $\iota$ -carrageenan mixtures, measured at room temperature were used for comparative purposes. All measurements were performed in triplicate.

#### **3.4.4 Electrophoretic mobility (zeta potential)**

Electrophoretic mobility of CPI-polysaccharide mixtures was determined as described in section 3.3.4, except pH was adjusted from 9.00 to 1.00 (in 1.00 pH increments) using a MPT-2 Multi-purpose autotitrator (0.05 M HCl, 1.0 M HCl, 0.1 M NaOH) (Malvern Instruments, Westborough, MA). Samples were prepared as described in section 3.4.3 for only the 20:1 CPI-alginate and CPI- $\iota$ -carrageenan-mixing ratio (0.05%, w/w total biopolymer concentration and 40 g total weight). CPI alone and GA solutions were also measured at corresponding concentrations as used in the mixed system. All measurements were made in triplicate.

#### **3.4.5 Coacervation at a higher total biopolymer concentration**

In preparation for solubility testing experiments were conducted on CPI-polysaccharide mixtures to ensure that associative phase behaviour was occurring at a higher total biopolymer concentration (1.0%, w/w). Samples were prepared at a 20:1 CPI-alginate and CPI- $\iota$ -carrageenan mixing ratio as in section 3.3.3, except at a total biopolymer concentration of 1.0% (w/w), and at pH 4.50 (adjusted with 0.1 M HCl). Associative phase behaviour was determined according to methods adapted from Koh and Tucker (1988). In brief, samples were allowed to mix for 30 min, and were held static for an additional 10 min at room temperature. Samples were then centrifuged (Sorvall Inc., Norwalk, CT) at 270 x g for 10 min, and then held at 4°C for 20 h to facilitate precipitation. The solutions were centrifuged again (270 x g) for 20 min. The supernatant was decanted into a 10 mL graduated cylinder to determine its total volume, whereas the residual pellet was weighed. The pellet was subsequently dissolved in 0.1 M NaOH to a total volume of 10 mL. Protein levels within the supernatant and pellet were determined using a micro-Kjeldahl digestion and distillation system (Labconco Corp., Kansas City, MO) according to AOAC Official Method (960.52) (%N x 5.70). Carbohydrate levels were determined using a phenol-sulphuric acid colorimetric test (Dubois et al., 1956). Briefly samples from the pellet and supernatant (2 mL) were

transferred to a test tube (1.8 cm x 15 cm) containing 1 mL of 5% (w/v) phenol solution. Five millilitres of concentrated H<sub>2</sub>SO<sub>4</sub> was then added to the test tube. Samples were left to stand for 10 min and then placed in a water bath held at (25°C) for 15 min. Sample absorbances were read at 490 nm. Sample absorbances were compared to standard curves prepared using alginate or ι-carrageenan solutions at concentrations ranging from 0.00047% and 0.025% (w/w). All measurements were made in triplicate.

#### **3.4.6 Solubility**

Percent protein solubility was determined in admixtures of CPI-alginate and CPI-ι-carrageenan at a mixing ratio of 20:1, total weight of 20 g and total biopolymer concentration of 1.0% (w/w), and at pH values of 7.00, 5.85, 5.41, and 4.50 for the CPI-alginate mixture and 7.00, 6.17, 5.85, and 4.50 for the CPI-ι-carrageenan system and pH values of 7.00, 6.17, 5.85, 5.41 and 4.50 for CPI. After pH adjustment, samples were stirred for 1 h, and then centrifuged at 16,700 x g for 20 min at room temperature. Supernatant was filtered (13mm, 0.2µm nylon syringe, Fisher Scientific, Ottawa, ON). Protein percent was determined by micro-Kjeldahl (calculations performed using eq. 1). All measurements were performed in triplicate.

#### **3.4.7. Statistics**

Statistical analysis of data collected during this study was conducted as outlined in section 3.3.7.

## **CHAPTER 4.0 RESULTS AND DISCUSSION**

### **4.1 Associative behaviour between pea proteins and gum Arabic (Study 1)**

#### **4.1.1 Proximate analysis**

Proximate analysis of PF showed 7.80% moisture, 21.70% protein (%N x 6.25), 1.00% lipid, 65.26% carbohydrate, and 4.16% ash. The proximate composition of the GA powder was 9.56% moisture, 5.19% ash, 85.25% carbohydrate; protein and lipid levels were considered negligible. Liu et al. (2009) found that GA contained 0.86% protein and 0.11% lipid. Carnovale et al. (1988) reported comparable levels of protein (22-24%) and slightly higher levels of moisture (10-11%) than those found in the present study for *Pisum sativum*. Marcone et al. (1998) found results for *Pisum sativum* that further supported the values obtained in this study with 10.47% moisture and 18.83% protein reported. Both Carnovale et al. (1988) and Marcone et al. (1998) performed their analysis on seeds.

#### **4.1.2 Preparation and characterization of pea protein isolates**

Pea flour contains a relatively low amount of total crude protein (21.70%). Using the method of Bora et al. (1994) for salt/pH protein extraction, a total mass from the three isolates of 10.61 g was recovered (giving a 19.56% protein recovery rate for the extraction process). Protein content of the Lg, Vn and mixed fractions recovered was found to be 96.12%, 94.45% and 92.03%, respectively as determined by the Bradford dye-binding method. Although protein levels were used further on an 'as is basis', it is important to note that protein determination by the Bradford-dye binding assay could lead to an over or under estimation of the true value, as it uses BSA for the standard curve rather than the protein of interest (i.e., absorbance may vary somewhat with different types of protein) (Runyon et al., 1993). The Bradford dye-binding method was used because of its sensitivity and low protein detection limits. It is reasonable to assume that the samples prepared by pH/salt extraction would have some level of cross-



contamination due to co-precipitation, based on the nature of the extraction process (Croy et al., 1980; Koyoro and Powers, 1987; O’Kane et al., 2004). Bora et al. (1994) also found co-precipitation for pea proteins (Lg and Vn) during pH/salt extraction of up to 30%.

Bora et al. (1994) used DEAE cellulose column chromatography for determining the relative percent cross-contamination in crude Lg and Vn pea isolates, based on earlier work by Grant and Lawrence (1964). Grant and Lawrence (1964) reported that Vn eluted from a DEAE cellulose column when a phosphate elution buffer containing 0.15 M NaCl was used, however when a 0.07 M NaCl concentration was used the Vn remained adsorbed to the column. Bora et al. (1994) proposed that crude fractions analyzed by DEAE cellulose column chromatography employing salt concentrations in a step-wise manner would yield both the major fraction of one protein (Lg or Vn) and a minor contaminant of the other. The authors assumed that the separated fractions using this method were pure, and applied this procedure to determine the relative composition of the pea isolates. The authors, using similar extraction methods as the present research reported their crude Vn fraction to be comprised of ~90.9% Vn and ~9.1% Lg, and their crude Lg fraction to be comprised of 68.5% Lg and 31.5% Vn. In the present study, the mixed isolate contained ~82% Lg and ~18% Vn, whereas the crude Lg isolate contained ~84% Lg and ~16% Vn, and the crude Vn isolate contained ~92% Vn and ~8% Lg (Table 4.1). Comparable results were found between the present research and that of Bora et al. (1994), showing cross contamination of protein fractions when samples were exposed to a DEAE cellulose column. Bora et al. (1994) considered the total protein eluted in the presence of 0.15 M NaCl to be Vn and with 0.40 M NaCl present to be Lg. Although the average percent recovery of material eluted from the column (relative to the original protein weight added) was relatively consistent between batches for each isolate, the ratio of major and minor components (Lg/Vn) varied between batches (Table 4.1). For instance, there was a ~2-fold difference in the Lg/Vn ratio for the mixed isolate between batches, and ~1.3 and ~3.3-fold differences between batches for the crude Lg and Vn isolates, respectively. These differences might be attributed to: a) a heterogeneously dispersed sample loaded onto the column; and b) varying levels of

absorbed protein on the stationary phase (i.e., the majority of the proteins added to the column remained during the chromatographic process).

Table 4.1. Percent Lg, Vn and average protein recovery within the mixed, Lg and Vn crude isolates, as determined by low pressure liquid chromatography with a DEAE stationary phase.

Protein	Percent Lg (or Vn) within each crude fractions*		Average recovery (%)**	
	Batch 1	Batch 2	Batch 1	Batch 2
<b>Mixed Isolate</b>				
Lg	77.46 ± 5.83	87.30 ± 1.16	42.65 ± 0.36	38.99 ± 0.33
Vn	22.54 ± 5.83	12.70 ± 1.16		
Ratio (Lg/Vn)	3.44	6.87		
<b>Crude Legumin</b>				
Lg	85.22 ± 0.15	82.08 ± 2.34	65.69 ± 3.25	59.83 ± 1.75
Vn	14.79 ± 0.15	17.93 ± 2.34		
Ratio (Lg/Vn)	5.76	4.58		
<b>Crude Vicilin</b>				
Lg	11.60 ± 2.23	3.76 ± 0.11	42.94 ± 2.81	43.24 ± 0.78
Vn	88.40 ± 2.23	96.25 ± 0.11		
Ratio (Lg/Vn)	0.13	0.04		

Notes:

\*Percent Lg and Vn found within each crude isolate was based on the weight ratio of collect fractions after step-wise elution with 0.15 M NaCl (Vn) and 0.4 M NaCl (Lg).

\*\*Average % recovery was determined by dividing the combined weights of the major and minor protein fractions collected, by the original weight of the crude isolates.

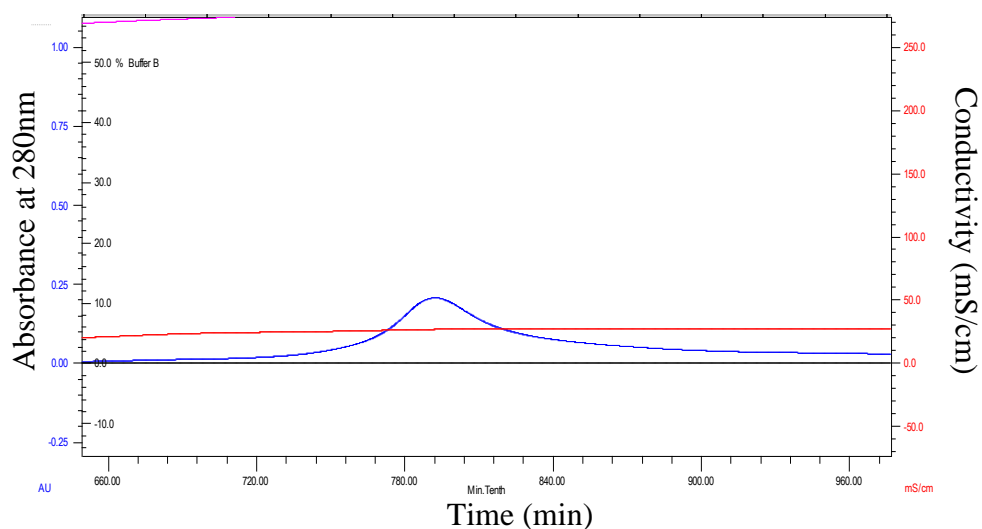
\*\*\* Data for each batch represent the mean ± one standard deviation of duplicate runs. Batch 1 and 2 refer to different crude isolates.

Figure 4.1 shows a typical chromatogram for both the Vn and Lg fractions. In the case of Vn, only one broad peak was observed corresponding to proteinaceous

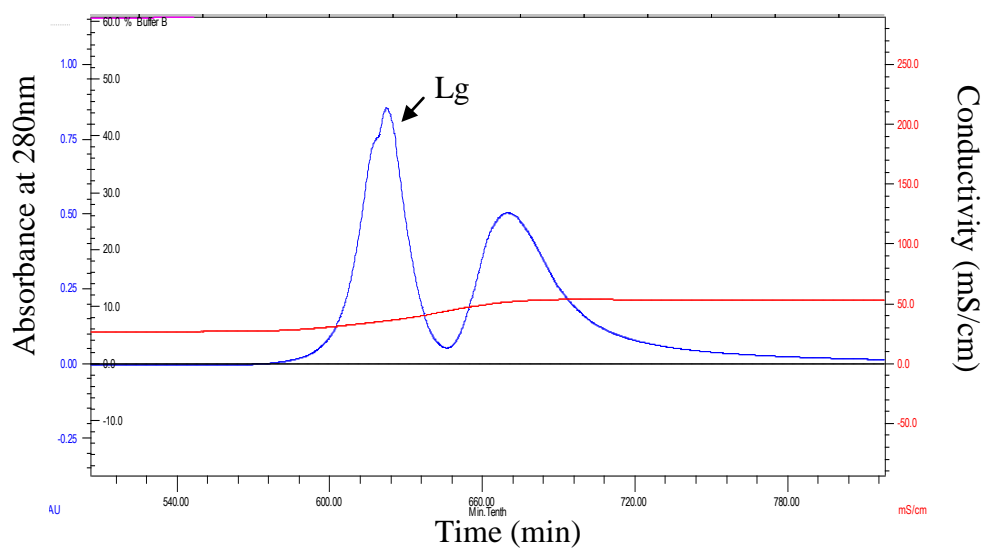
material as determined by the Bradford-dye binding method (Figure 4.1a). In contrast, the Lg isolate was comprised of two peaks, with only the first peak being associated with proteinaceous material (Figure 4.1b). Marcone et al. (1998) reported a peak containing no protein which was eluted from a gel filtration column at or near the void volume. The authors reported that seed globulin fractions often contain turbid material with strong UV-absorption and hypothesized this peak could contain nucleic acids and other unidentifiable high molecular weight compounds (this peak was also found to contain carbohydrates). In the present study, a similar non-proteinaceous peak/fraction was seen using an ion exchange column when the mobile phase contained a high NaCl concentration (0.40M). Marcone et al. (1994) also reported a similar non-proteinaceous peak for soybean 11S globulin. However, the presence of non-protein related UV-absorbing peaks are not always present within globulin extracts. Marcone et al. (1997) studying the 12S seed globulin from mustard seed (*Brassica alba*) found only one UV-absorbing peak associated within the fraction rich in protein. Major isolates collected from LPLC using the crude Lg and Vn isolates were deemed the 'enriched Lg and Vn isolate fractions'. Based on the Bradford-dye assay, protein levels in the enriched Lg and Vn fractions were found to be ~100% and ~97%, respectively.

Native- and SDS (non-reducing)-PAGE was used on PF, the crude isolates prepared by pH/salt extraction (e.g., mixed isolate, Lg and Vn), and the Lg and Vn enriched isolates to access changes in purity and for molecular weight determination. Native-PAGE allows for changes in conformation to be determined, but has limitations in determining size (Marcone et al., 1998). Figure 4.2 shows a typical native-PAGE gel of PF and the various pea protein isolates. Despite PF containing a variety of albumin and globulin proteins, only one major band was observed in the native gel (lane 1). This may be attributed to: a) size (70-700 kDa) and concentration (50-100 ng) detection limits and/or b) poor separation of the major proteins (Lg 350-400 kDa and Vn 150 kDa). In the case of the mixed (lane 2), and both the crude (lane 3) and enriched Lg (lane 4) isolates one major and one minor band was found. Whereas for both crude and enriched Vn isolates, one diffuse band was observed (lanes 5,6). It is hypothesized that based on similar motilities of the major band found in the PF and all protein fractions, that Lg and Vn proteins had difficulty separating from one another within the gel. The

size of the two globulin proteins were presumed to be similar so that resolution between bands was not achieved. In addition, all fractions had similar net charges with pI values ranging between 4.70 and 5.30 (see section 4.13).



**(a) Vicilin**



**(b) Legumin**

Figure 4.1. LPLC-DEAE chromatograms of pea vicilin (a) and pea legumin (b) isolates employing a phosphate buffer (35mM) and sodium chloride (0.15M for vicilin and 0.40M for legumin) mobile phase.

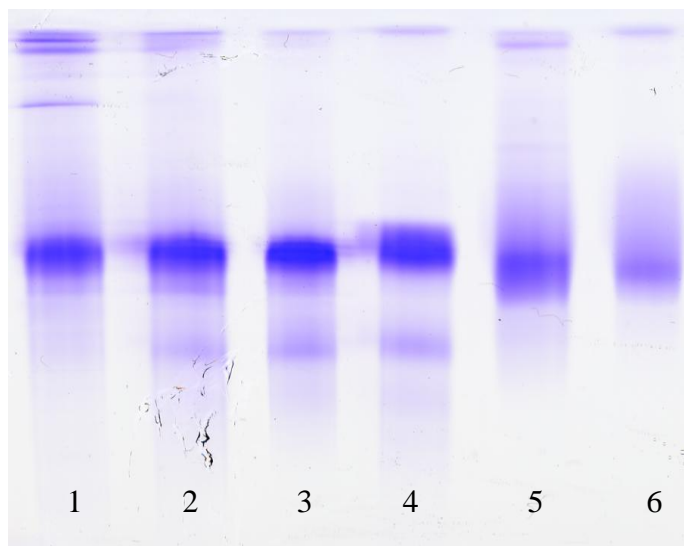


Figure 4.2. Native-PAGE (1  $\mu$ L of 3 mg mL<sup>-1</sup>) using 4-15% gradient phastgels. Lanes: (1) PF, (2) mixed isolate, (3) crude Lg isolate, (4) enriched Lg isolate, (5) crude Vn isolate, and (6) enriched Vn isolate.

SDS-PAGE on the same protein fractions under non-reducing conditions showed the presence of multiple major and minor bands reflecting the heterogeneous nature of the pea protein isolates (Figure 4.3). Pea flour (lane 2), the mixed isolate (lane 3) and the crude Lg isolate (lane 4) all contained major bands at approximately 86, 67, 59, 47, 41, 31 and 19 kDa. Upon enrichment of the crude Lg isolate, major bands were found at 59 and 41 kDa intensity (lane 5). Because SDS-PAGE was performed under non-reducing conditions, it is hypothesized that the 59 kDa band was associated with the  $\alpha,\beta$  pair subunit of the Lg protein. The major bands (59 and 41 kDa) remained prominent within the PF, mixed isolate and crude and enriched Lg isolates, whereas others progressively diminished, as the system was enriched (lanes 2-5). These bands were also absent within the crude and enriched Vn isolates (lanes 6,7). The identity of the other major and minor bands within the crude and enriched Lg isolates (lanes 4,5) are unknown. The heterogeneous nature in pea Lg is not well established in the literature (Gatehouse et al., 1980). However, it may arise as a result of cross-contamination, post-translational modification and/or storage artifacts in samples purified from dry seeds. Gatehouse et al. (1980) reported that for SDS-PAGE (non-reducing) Lg samples, major bands were found at 60, 38 and 31-29 kDa.

In the case of the crude Vn isolate, major bands were evident at 68, 48 and 32 kDa (lane 6), whereas once enriched, major bands were present at 48 and 32 kDa (lane 7). Multiple minor bands were also observed in both the crude and enriched Vn isolates at relatively similar intensities. The higher molecular weight band found in the crude Vn is presumed to reflect cross-contamination from other proteins (lane 6). In literature (under non-reducing conditions), O’Kane et al. (2004) reported major bands for pea vicilin at 50, 33, 30, 19, 16, 14 and 12.5 kDa; whereas Spencer et al. (1983) reported major bands at 50, 34, 30, 25, 18, 14, 13 and 12 kDa.

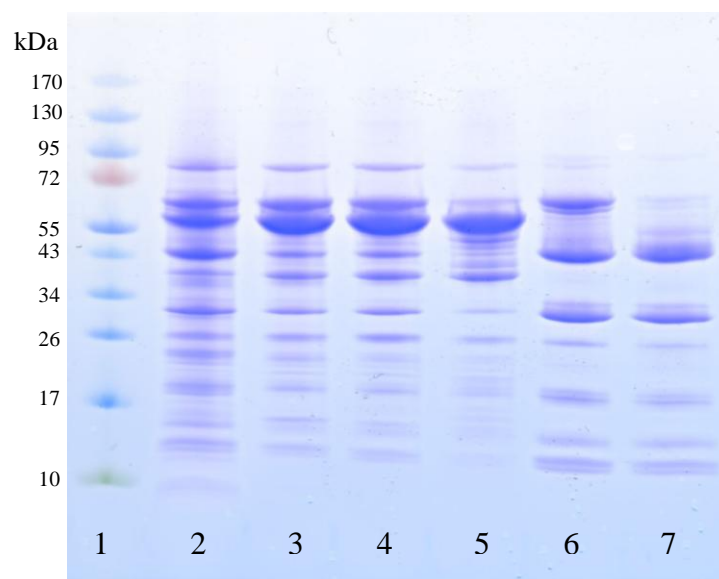


Figure 4.3. SDS-PAGE (non-reducing) ( $1 \mu\text{L}$  of  $2 \text{ mg mL}^{-1}$ ) using 8-25% gradient phastgels. Lanes: (1) standard, (2) PF, (3) mixed isolate, (4) crude Lg isolate, (5) enriched Lg isolate, (6) crude Vn isolate, and (7) enriched Vn isolate.

#### 4.1.3 Associative phase behaviour of pea protein isolates with gum Arabic

Associative phase behaviour as a function of pH was investigated by optical density analysis for all protein isolates (i.e., isolate, crude and enriched Lg and, crude and enriched Vn) with GA at a 2:1 protein:GA mixing ratio. This biopolymer ratio was

selected based upon earlier work by Liu et al. (2009) who investigate the coacervation behaviour of a pea protein isolate with GA.

*(a) Admixtures of crude and enriched Lg with GA*

Optical density profiles as a function of pH in systems containing only protein (crude and enriched Lg) are given in figure 4.4(a,b). Significant scattering intensities were seen for both the crude and enriched Lg systems alone over a pH range of 3.20 to 4.80, and 3.20 to 6.00, respectively, reaching a maximum optical density at 600 nm of ~0.400. The rise in scattering intensity is thought to result from an increase in protein-protein particle size as the degree of aggregation increases near the pI. In contrast, no scattering intensity was seen over the complete pH range in the case of GA (not shown). Based on electrophoretic mobility measurements as a function of pH, the zeta potential (0 mV) (or pI) coincided with pH 4.80 and 4.95 for the crude and enriched Lg isolates, respectively, and 1.85 for GA (Figure 4.4c,d).

For mixed protein-GA systems at solution pH greater than the pI of the major proteins, both biopolymers carry a similar net charge and are considered to be co-soluble. Biopolymers are presumed to be spatially separated as a consequence of the dilute nature of the medium and their charge repulsion. Aggregation of self-similar biopolymers by segregative processes is also expected, particularly in the case of the Lg proteins where hydrophobic interactions are thought to lead to the formation of small protein-protein aggregates. The extent of which is considered to be limited due to the strong electrostatic repulsive forces present in the system which resulted in minimal optical density readings at pHs greater than 3.87 and 4.18 for the crude Lg-GA and enriched Lg-GA systems, respectively, even though Lg took on a positive net charge at pH less than pI (4.80 and 4.95 for crude and enriched Lg, respectively). As pH decreases further, the protein's positive net charge increases and the electrostatic attraction to the anionic GA molecule leads to the formation of protein-polysaccharide complexes.

For the crude Lg-GA system, complex coacervation is thought to occur through two structure-forming events associated with the formation of soluble ( $\text{pH}_c$ ) and insoluble ( $\text{pH}_{\phi 1}$ ) complexes at pH 3.87 and 3.67, respectively (Figure 4.4a, Table 4.2). At pH lower than  $\text{pH}_{\phi 1}$ , the solution underwent a transparent-to-cloudy transition, as

evidenced by the significant rise in optical density up to a maximum of ~0.960, where significant crude Lg-GA interactions occurred ( $\text{pH}_{\text{opt}}$  3.50) (Figure 4.4a, Table 4.2). At pH less than  $\text{pH}_{\text{opt}}$ , the stability of the crude Lg-GA complexes was reduced due to the progressive protonation of the carboxyl group along the GA backbone during acid titration until reaching  $\text{pH}_{\phi 2}$  (pH 2.58) (Figure 4.4a, Table 4.2). A similar profile was evident for the enriched Lg-GA system, except the formation of soluble complexes occurred at a higher pH (4.18) than with the crude isolate, and the maximum absorbance at  $\text{pH}_{\text{opt}}$  was greater (1.100) (Figure 4.4b, Table 4.2). No significant differences were found between the crude Lg-GA and enriched Lg-GA systems at other critical pH values ( $\text{pH}_{\phi 1}$ ,  $\text{pH}_{\text{opt}}$ , and  $\text{pH}_{\phi 2}$ ) (Table 4.2). It was hypothesized that the enriched isolate may have a higher amount of hydrophilic amino acids present, which could improve the stability of soluble complexes through the secondary effects of hydrogen bonding. The overlap within the Lg isolate and Lg-GA systems within the pH range of ~3.20-3.80 suggests that complexes formed involve GA molecules with small aggregated clusters of Lg rather than individual proteins. Liu et al. (2009) reported a  $\text{pH}_c$  dependence on biopolymer mixing ratio for a pea protein isolate-GA system. This dependence indicates that protein-protein aggregates became larger with increasing biopolymer concentration until reaching a critical size, as the aggregate size increases  $\text{pH}_c$  can be detected earlier. The addition of GA molecules to the protein systems acted to lower the pH where the isolates were electrically neutral (zeta potential = 0 mV), coinciding to 4.05 and 3.95 for the crude Lg-GA and enriched Lg-GA systems, respectively. For Lg, similar turbidity and electrophoretic mobility profiles were expected between the crude and enriched isolates due to similar protein patterns observed by electrophoresis.

A significant shoulder appeared in both the mixed crude Lg-GA and enriched Lg-GA turbidity profiles at pHs less than 3.20. Similar findings were reported by Liu and co-workers (2009, 2010a,b) for a pea protein isolate-GA mixture. Liu et al. (2010b) hypothesized this shoulder to be the result of hydrophobic interactions that stabilize the protein-protein aggregates under acidic conditions that are involved within the electrostatically bound complexes. The authors reported the shoulder to become more pronounced at a higher temperature (60°C) using similar optical density testing, which corresponded to the presence of larger structures at pH 3.10 relative to those formed at



lower temperatures, or protein alone systems as observed by confocal scanning light microscopy.

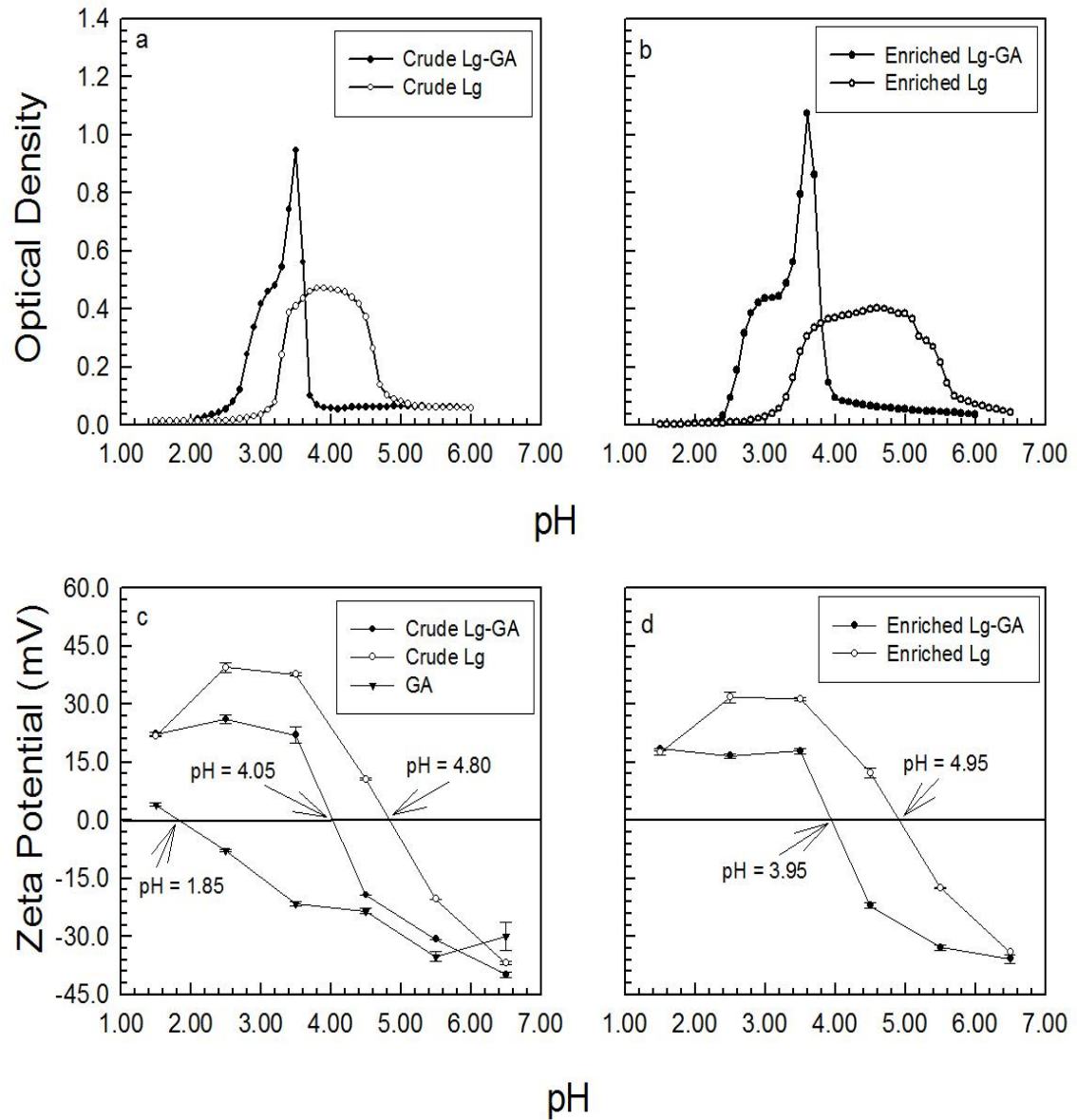


Figure 4.4. Mean optical density curves and zeta potentials for protein alone and mixed crude (a, c) and enriched (b,d) Lg-GA systems as a function of pH ( $n = 3$ ). Zeta potential data represents the mean  $\pm$  one standard deviation ( $n = 2$ ).

Table 4.2. Critical pH transition points ( $\text{pH}_c$ ,  $\text{pH}_{\phi 1}$ ,  $\text{pH}_{\text{opt}}$  and  $\text{pH}_{\phi 2}$ ) corresponding to structure-forming events within a optical density pH-acid titration for a mixed pea protein isolate, crude and enriched legumin (Lg), and crude and enriched vicilin (Vn) isolates with GA at a 2:1 protein-GA mixing ratio (values represent the mean  $\pm$  one standard deviation). Values denoted by different letters indicate significant differences ( $p < 0.05$ ).

Protein fraction	$\text{pH}_c$	$\text{pH}_{\phi 1}$	$\text{pH}_{\text{opt}}$	$\text{pH}_{\phi 2}$
Mixed-GA	$3.89 \pm 0.02^a$	$3.64 \pm 0.08^d$	$3.47 \pm 0.06^e$	$2.62 \pm 0.06^f$
Crude Lg-GA	$3.87 \pm 0.06^a$	$3.67 \pm 0.03^d$	$3.50 \pm 0.05^e$	$2.58 \pm 0.03^f$
Enriched Lg-GA	$4.18 \pm 0.18^b$	$3.87 \pm 0.05^d$	$3.66 \pm 0.08^e$	$2.42 \pm 0.02^g$
Crude Vn-GA	$4.05 \pm 0.05^{ab}$	$3.61 \pm 0.08^d$	$3.47 \pm 0.06^e$	$2.51 \pm 0.03^{fg}$
Enriched Vn-GA	$5.01 \pm 0.01^c$	$3.72 \pm 0.01^d$	-	-

*b) Admixtures of crude and enriched Vn with GA*

Optical density curves as a function of pH for both crude and enriched Vn, are shown in figure 4.5(a,b). Optical density as a function of pH was found to rise from pH ~4.80-4.60 then plateaued until pH 3.2 followed by decreasing to baseline values at pH 2.80 for the crude Vn isolate, whereas the enriched Vn isolate increased from pH ~6.20-5.80 then plateaued until pH 4.60 when it decreased sharply until pH 4.20. For both systems, maximum optical density was found to occur near 0.200. Electrophoretic mobility as a function of pH revealed the pI of the crude and enriched Vn isolates to be 4.70 and 5.30, respectively (Figure 4.5a,b). It is presumed that differences in optical density profiles and pI values reflect differences in protein composition after enrichment, where SDS-PAGE indicated the absence of a 68 kDa protein band post-column separation.

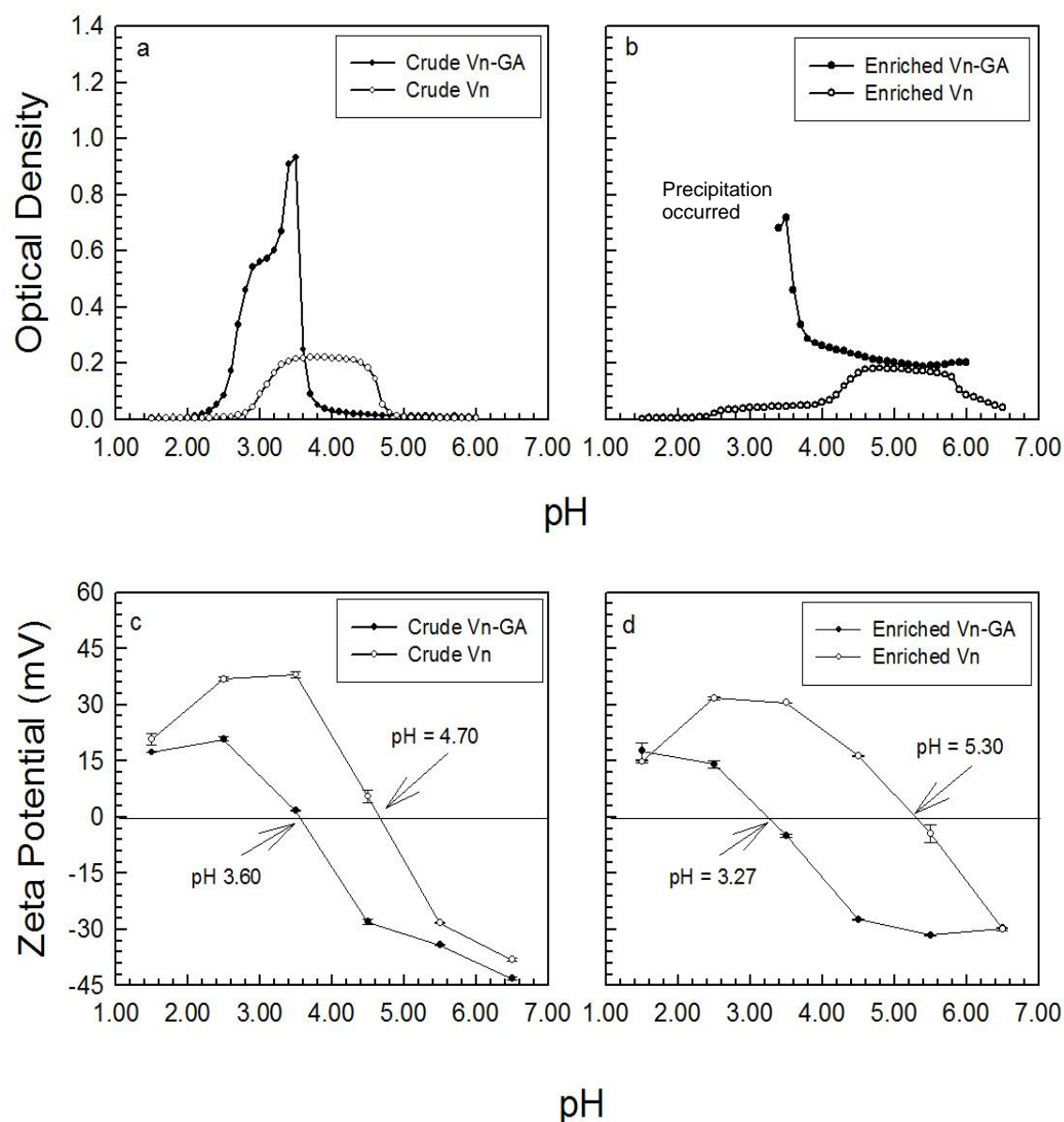


Figure 4.5. Mean optical density curves and zeta potentials for protein alone and mixed crude (a,c) and enriched (b,d) Vn-GA systems as a function of pH ( $n = 3$ ). Zeta potential data represent the mean  $\pm$  one standard deviation ( $n = 2$ ).

Optical density curves for Vn (crude and enriched)-GA systems as a function of pH, are shown in figure 4.5(a,b). In the crude Vn-GA mixture, soluble and insoluble complexes were formed at pH 4.05 and 3.61, respectively (Figure 4.5a, Table 4.2).

Optical density increased significantly up to a maximum of 0.960 at pH 3.47 ( $\text{pH}_{\text{opt}}$ ), before declining until pH 2.51 ( $\text{pH}_{\phi_2}$ ) after which complexes disassociated (Table 4.2). Overlap between the protein optical density curve and that of the mixed at pH less than 3.60, and the presence of a shoulder at pH less than 3.20 suggests a similar mechanism for coacervation formation with GA as Lg. That is complexes form between small Vn-Vn clusters and GA chains; and are further stabilized by hydrophobic interactions within the protein aggregates. Optical density curves involving the enriched Vn-GA system behaved quite differently (Figure 4.5b). For instance, soluble complexes formed at much higher pH levels (5.01) than within the crude sample, whereas insoluble complexes formed at similar pHs (Table 4.2). However, at pH less than  $\text{pH}_{\phi_1}$  the system began to precipitate at a much faster rate than in the crude sample preventing any reliable information relating to  $\text{pH}_{\text{opt}}$  and  $\text{pH}_{\phi_2}$  from being obtained. Differences in initial complex formation and behaviour in solution below  $\text{pH}_{\phi_1}$  may reflect changes in protein composition as the 68 kDa band was removed upon enrichment by FPLC. The addition of GA also caused the pH where complexes became electrically neutral (zeta potential = 0 mV) to shift from 4.70 to 3.60 for crude Vn, and 5.30 to 3.27 for enriched Vn (Figure 2.5c,d). Based on the NaCl concentration needed to elute Lg from the DEAE column it was expected that Lg would have a greater negative charge than Vn, this is indicated by Lg's lower pI.

*c) Admixtures of the mixed pea protein isolate with and without GA*

Optical density profiles as a function of pH for mixed isolate solutions were found to increase from pH 4.60 to 4.40 then plateau until 3.20 before decreasing from pH 3.20 to 2.60, with maximum optical density occurring at ~0.200 (Figure 4.6a). Optical density curves and zeta potential data for the mixed pea protein isolate-GA mixture followed a similar profile as the crude Lg-GA and Vn-GA systems. For the mixed isolate-GA system, soluble and insoluble complexes were formed at pH 3.89 and 3.64, respectively; with optimal interactions ( $\text{pH}_{\text{opt}}$ ) occurring at pH 3.47 and the dissolution of complexes at pH 2.62 ( $\text{pH}_{\phi_2}$ ) (Figure 4.6a, Table 4.2) Electrophoretic mobility as a function pH indicated a pI of 4.93 for the mixed isolate, and the complex being electrically neutral at pH 3.80 (Figure 4.6b). Similar to the other crude mixtures, a

pronounced shoulder was found at pH less than 3.20 and, overlap between the protein alone and the mixed curves suggesting similar complexation mechanisms occurring. Results indicate that the level of enrichment of either Lg or Vn proteins had little effect on the complexation behaviour over preparation of a simple crude protein isolate.

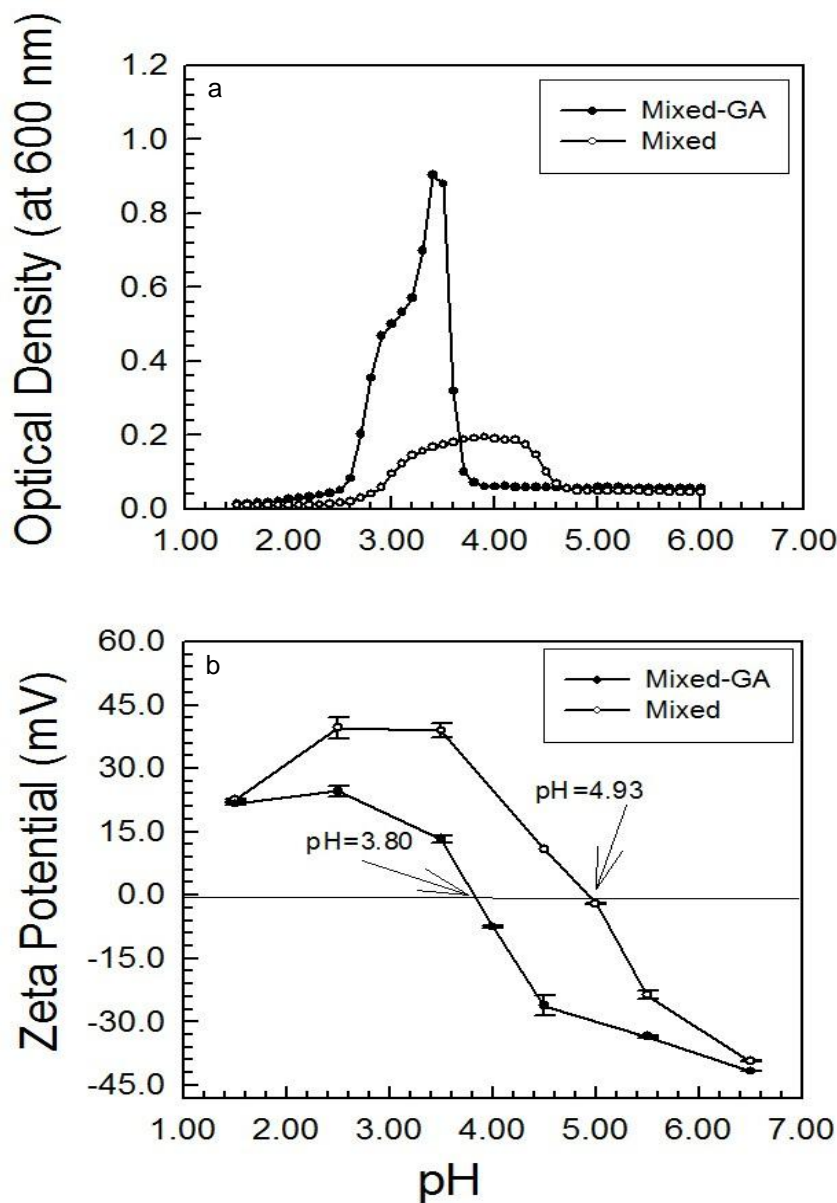


Figure 4.6. Mean optical density curve (a) and zeta potential (b) for a mixed pea protein isolate and a mixed pea protein isolate-GA system as a function of pH ( $n = 3$ ). Zeta potential data represent the mean  $\pm$  one standard deviation ( $n = 2$ ).

#### **4.1.4 Complexation-induced changes to the tertiary conformation of enriched Lg and Vn isolates**

Changes to the tertiary conformation of both the enriched Lg and Vn isolates during complexation were investigated by fluorescence emission spectroscopy at pHs corresponding to the presence of soluble (pH 4.00) and insoluble (pH 3.70 for legumin and pH 3.60 for vicilin) complexes. Fluorescence intensity (FI) was also measured at pH 5.50 for the enriched Lg and Vn fractions and GA, under conditions where biopolymers remained co-soluble. In the case of GA, the FI was negligible (FI less than 0.02) over the wavelength range (300-400 nm) (not shown), relative to the homogenous protein and mixed systems. The fluorescence spectrum of a protein is highly sensitive to the position of tyrosine and tryptophan in the protein structure and changes in the spectrum can give an indication of changes to protein folding, induced by environmental factors such as, pH, salt or temperature (Wrolstad et al., 2005). Aluko and Yada (1995) found that masking protein charge can lead to increased hydrophobicity of the protein based on a high FI at low pH values. In the case of Lg, no significant differences in FI were found between the enriched Lg isolate and the Lg-GA system at either pH 4.00 or 3.70 (Table 4.3, Figure 4.7). In the case of Vn, complexation with GA resulted in an increase in FI intensity relative to Vn alone ( $p < 0.05$ ) at pH 4.00 and 3.60 (Table 4.3, Figure 4.8), indicating a complexation induced conformational change in Vn structure. Changes in structure upon complexation have also been previously reported. Chourpa et al. (2006) found that the addition of GA led to changes in the secondary structure of pea globulin and wheat  $\alpha$ -gliadin. Alone, the globulin protein was high in  $\beta$ -sheets and random coils, whereas  $\alpha$ -gliadin proteins were high in  $\alpha$ -helices and random coils. Upon complexation with GA, both  $\alpha$ -helices and random coils were favoured above pH 3.00. While at pH less than 3.00  $\beta$ -sheets increased for both systems. Girod et al. (2004) found that poly(L-lysine) takes on a right-handed  $\alpha$ -helical conformation upon complexation with  $\iota$ -carrageenan.

Table 4.3. Fluorescence intensity of enriched Lg and Vn isolates, and enriched Lg-GA and Vn-GA systems at solvent pH corresponding to where soluble and insoluble complexes were present. Data represent the mean values  $\pm$  one standard deviation (n=2).

pH	Maximum fluorescence intensity (arbitrary units a.u.)		
	Protein alone	Protein-GA mixture	
Legumin			
4.00	1.675 ± 0.035	1.695 ± 0.021	(Soluble complexes)
3.70	1.650 ± 0.028	1.610± 0.014	(Insoluble complexes)
Vicilin			
4.00	0.686 ± 0.026	0.889 ± 0.013	(Soluble complexes)
3.60	0.571 ± 0.031	0.974 ± 0.023	(Insoluble complexes)

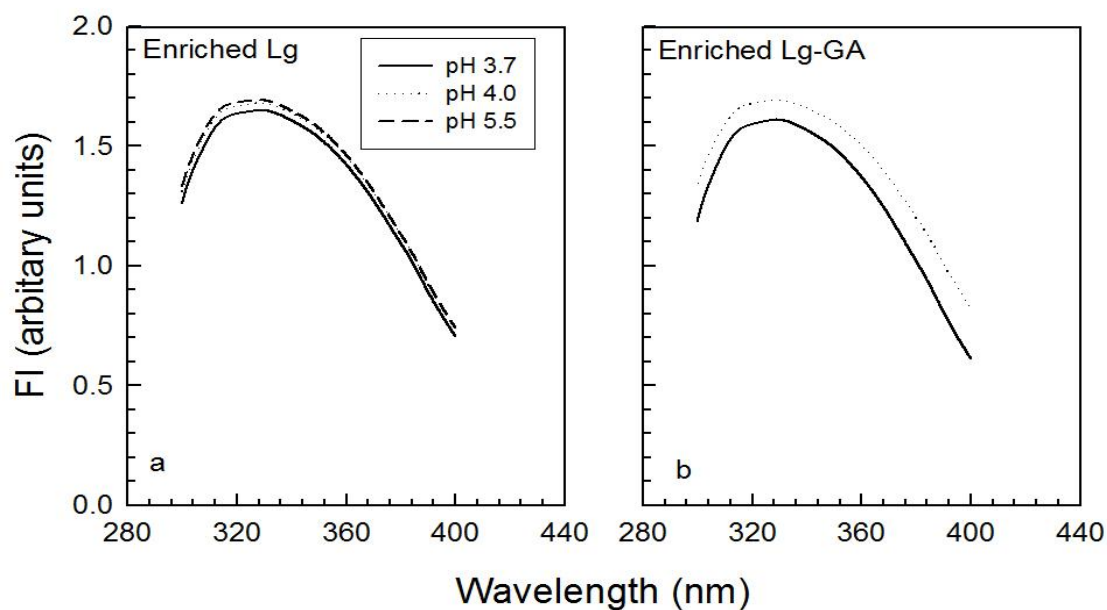


Figure 4.7. Mean fluorescence intensity for enriched Lg (a) and enriched Lg-GA (b) systems as a function of pH (n = 2).

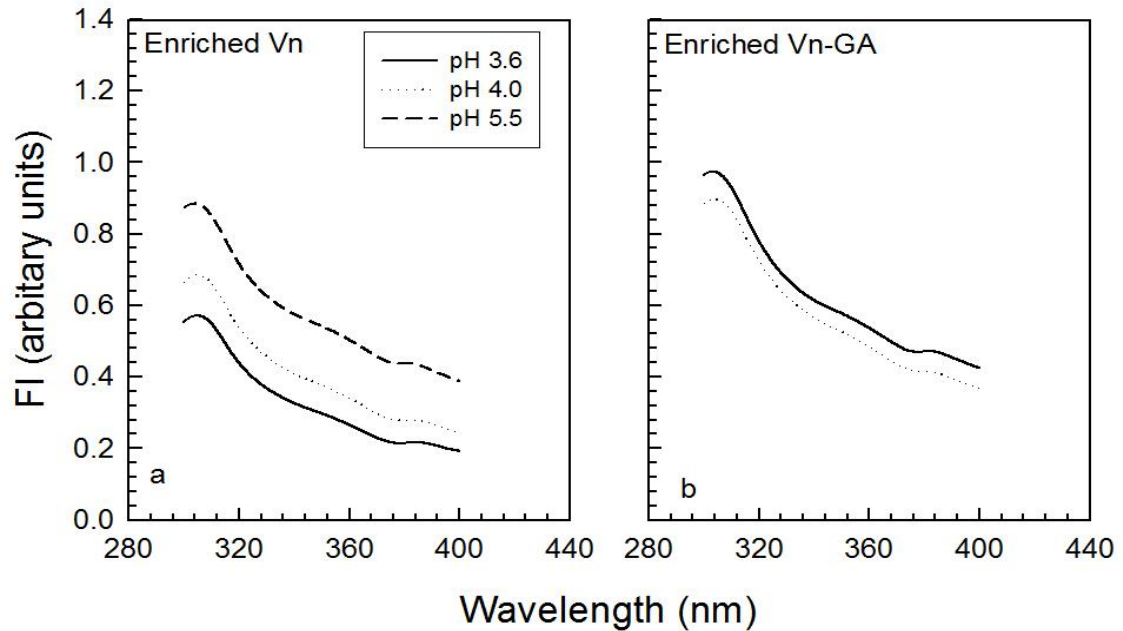


Figure 4.8. Mean fluorescence intensity for enriched Vn (a) and enriched Vn-GA (b) systems as a function of pH ( $n = 2$ ).

#### 4.1.5 Solubility

Protein solubility was investigated for the mixed, crude Lg and crude Vn isolates with and without GA as a function of pH at a higher total biopolymer concentration than was used in the optical density studies (1.00% and 0.05%, w/v, respectively) (Figure 4.9). Solubility at most pH values (2.00, 4.00, 5.00, 6.00, 6.50 and 10.00) did not significantly change in the presence of GA when compared to the isolate alone. However, at pH 3.0 for all isolates the mixed systems (containing GA) had significantly lower solubilities when compared to the isolate alone. At pH 3.50 both Lg-GA and mixed-GA systems were significantly different when compared with their isolate alone. In all cases, no solubility was observed at pHs ranging from 4.00-5.00. Within the pH range of 3.50 to 6.50 protein and protein-GA systems were almost entirely insoluble. At pH 7.00 only the Lg-GA system was significantly different from the isolate alone. At pH 8.00 both Vn-GA and mixed-GA were significantly different for the isolates alone, while at pH 9.00 only Vn-GA was significantly different from the isolate alone. Differences at pH 7.00, 8.00 and 9.00 may be due to over- or underestimation in protein



percents by Bradford analysis. Overall the Lg was found to be more soluble under acidic conditions than the Vn isolate, whereas the opposite was true under basic conditions. For instance, at pH 3.00 Lg was 93.3% soluble which was significantly different from Vn which was only 49.5%. At pH 7.00, Vn was 85.9% soluble this was significantly different when compared with Lg, which was 57.9%. Koyoro and Powers (1987) found Lg, Vn and mixed fractions were insoluble at pH 5 and 6, but became soluble at pH 4. Harnsilawat et al. (2005) reported that the solubility of  $\beta$ -lactoglobulin at pHs 3, 4 and 5 decreased in the presence, and at increased levels of sodium alginate. At higher pHs (6 and 7), the authors found the solubility was unaffected by the presence of alginate.

Liu et al. (2010b) reported minimal solubility for a pea protein isolate system between pH 5.00-6.00, whereas in a mixed isolate-GA mixture the minimum broadened was reported at pH 3.50-6.00. At the minimum for the protein, solubility was reported to be close to 20%, which is much higher than that found in these studies. Liu et al. (2010b) used a Kjeldahl digestion-distillation method for determining protein content, which measures the total nitrogen content in the material and assumes all nitrogen present is associated with proteins. As a consequence their findings may have overestimated the true protein content if free amino acids and peptides were present. However, in the present study, the Bradford dye-binding assay may also have underestimated the true protein levels. The binding assay is based on electrostatic interactions between the negatively charged Coomassie Blue dye molecule and the positively charged patches (basic amino acids primarily arginine, lysine and histidine) on the protein's surface (Van Kley and Hale, 1977). Colour change occurs by a shift from the red form of the Coomassie dye to the blue form upon binding to the protein; therefore the number of positive charges on the protein is roughly proportional to the amount of dye ligands bound to that protein (Bradford, 1976).

#### **4.1.6 Summary**

In this study, the effect of both pH and protein enrichment on the complexation behaviour of pea Lg or Vn isolates with GA was investigated. In all cases, complex formation occurred over a narrow pH range where biopolymers exhibited opposing

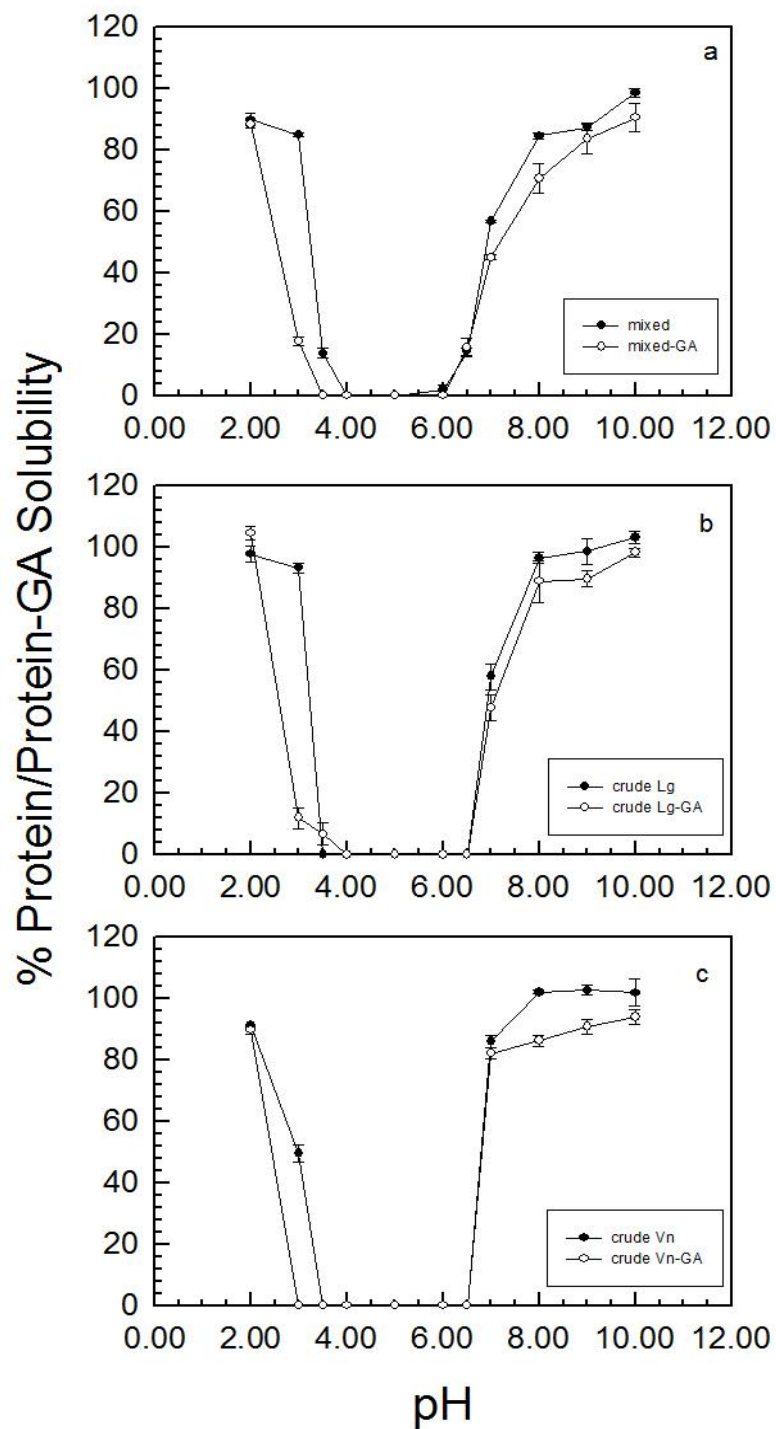


Figure 4.9. Mean protein solubility (%) for the mixed, Lg and Vn isolates with and without GA as a function of pH. Data represent the mean  $\pm$  one standard deviation ( $n = 3$ ).

charges, and followed a similar mechanism of association. Initially, small protein protein clusters formed at pH values greater than  $\text{pH}_c$ , followed by the formation of soluble and insoluble higher ordered structures in the presence of GA. The presence of protein-protein clusters within the electrostatically bound protein-protein-GA complex provided additional stability at pH less than 3.00, probably due to hydrophobic interactions that help stabilize the aggregates. Overall, the coacervation behaviour of crude and enriched Lg-GA and Vn-GA systems were comparable to the crude mixed isolate-GA system, with minor differences found in the enriched Vn-GA system relative to the crude at only  $\text{pH}_c$  (formation of soluble complexes). The Vn-GA system also switched from a system where complexes remained suspended to one that precipitated at pHs less than  $\text{pH}_{\phi 1}$  upon enrichment. Changes to the protein conformation of the enriched Vn in the presence of GA was also evident, whereas no changes were observed in the case of Lg. Mixed systems were found to have low or no protein solubility between pH 3.50 and 6.00 and in general solubility for these systems was not found to be improved in comparison with the protein alone.

## **4.2 Associative phase separation involving canola protein isolate with both sulphated and carboxylated polysaccharides**

### **4.2.1 Proximate Analysis**

Ground canola seed, defatted meal and the canola protein isolate (CPI) were assessed for their protein, moisture, ash and lipid content according to standard AOAC methods. Carbohydrate content was based on a percent differential from 100%. In contrast, alginate and ι-carrageenan were analyzed for their moisture, ash and carbohydrate contents as lipid and protein levels were considered negligible. Ground canola seed was found to be comprised of 44.10% lipid, 3.37% moisture, 22.26% protein (%N x 5.70), 4.32% ash, and 25.96% carbohydrate. The defatted canola meal was found to contain 39.34% protein, 4.44% moisture, 1.88% lipid, 7.80% ash and 46.54% carbohydrate. This composition was similar to an industrial rapeseed meal reported by Chabanon et al. (2007), which contained 41.4% protein, 3.2% lipid and 7.6% ash. The prepared CPI was found to be comprised of 94.95% protein, 0.70% moisture, 0.32% lipid, 2.32% ash and 1.71% carbohydrate. In contrast, alginate powder was comprised of 11.95% moisture, 24.45% ash and 63.60% carbohydrate, whereas the ι-carrageenan powder contained 11.44% moisture, 24.80% ash and 63.76% carbohydrate. Uruakpa and Arntfield (2005) used a commercial CPI which contained 87% protein, 0.7% lipid, 2% ash, 5.9% moisture and 4.4% carbohydrate. Biopolymer concentrations used in this study reflect the protein (CPI) or carbohydrate (alginate or ι-carrageenan) content rather than powder weight.

### **4.2.2 Preparation and characterization of the canola protein isolate**

A canola protein isolate was prepared from the defatted meal using a pH-salt extraction process, which raised protein levels from 39.34% to 94.45%. SDS-PAGE (non-reducing) of the canola meal revealed multiple major bands occurring at 13-15, 17, 25, 28, 51-57 kDa (Figure 4.10). Upon the addition of mercaptoethanol (reducing conditions), the higher (greater than 30 kDa) molecular weight bands disappeared, leaving bands at 10, 18-20, 25 and 28-30 kDa. Mercaptoethanol acts to reduce the number, or eliminate disulphide bonds stabilizing the larger subunits from some of the

canola proteins (i.e., cruciferin) (Berot et al., 2005; Uruakpa and Arntfield, 2006). Fainter minor bands were also seen throughout the gel reflecting the heterogeneous nature of the material (Figure 4.10).

Similar results were found for the isolate, where major bands under non-reducing conditions were identified at 13, 17-19, 27 and 50-56 kDa, and under reducing conditions at 10, 18-20, 25 and 28-30 kDa (Figure 4.10). Similarities between the band profiles of the meal and the isolate indicates that the pH/salt extraction acted to increase the total protein content rather than significantly change the protein profile. Similar findings for canola isolates have been reported in the literature. Aluko and McIntosh (2001) reported four major bands at 16, 18, 30 and 53 kDa under non-reducing conditions using isolates produced from *Brassica napus*. Uruakpa and Arntfield (2006) found major bands at 17, 21, 27 and 45 kDa under non-reducing conditions in a commercial canola protein isolate. Schwenke (1998) reported major bands at 18.5, 21.1, 26.8 and 31.2 kDa under reducing conditions for a *Brassica napus* isolate.

#### **4.2.3 Associative behaviour of CPI with alginate and ι-carrageenan**

In the present study, the effect of pH (1.50-7.00), weight mixing ratio (1:1 to 50:1) and polysaccharide-type (ι-carrageenan (sulphated); alginate (carboxylated)) on the formation of soluble and insoluble complexes, and surface charge was investigated in CPI and mixed CPI-polysaccharide systems. In the case of CPI solutions at concentrations corresponding to those used in the mixed systems, a rise in maximum optical density was observed as protein levels increased from 0.0250% (optical density ~0.340) to 0.0490% (w/w) (optical density ~0.920). These results were thought to be associated with a rise in the degree of CPI-CPI aggregation (Figure 4.11). In all cases, optical density curves were broad, spanning a pH range of ~3.50 to 6.50. The optical densities for alginate and ι-carrageenan as a function of pH were found to be negligible (not shown).

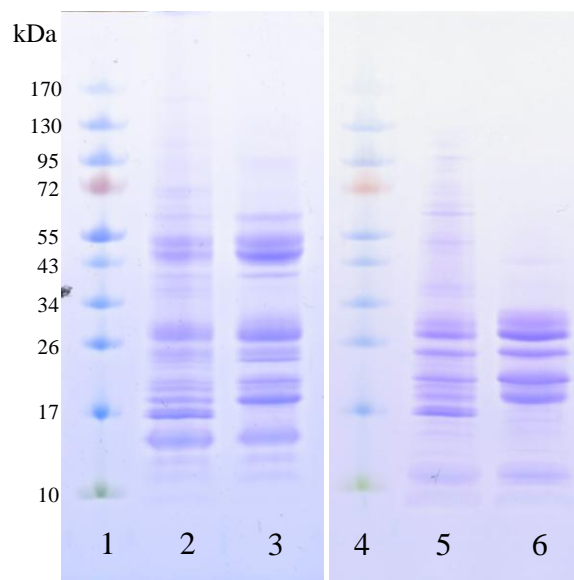


Figure 4.10. SDS-PAGE of the canola meal and isolate under non-reducing and reducing conditions. Lanes are as follows: (1,4) protein standard; (2) meal (non-reducing); (3) isolate (non-reducing); (5) meal (reducing); and (6) isolate (reducing).

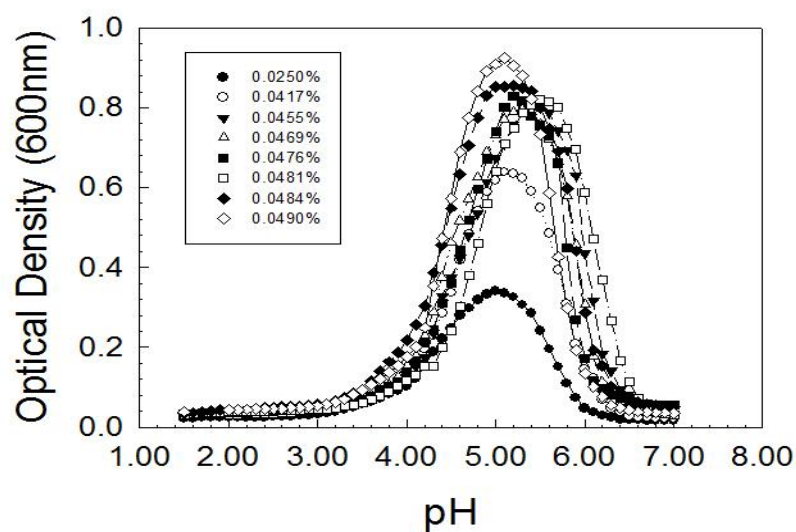


Figure 4.11. Mean optical density curves for CPI solutions as a function of pH and concentration (% , w/w) (n = 3).

In the presence of anionic polysaccharides as shown in Figure 4.12, CPI aggregation was significantly suppressed relative to CPI alone. The suppression of CPI-CPI aggregation due to electrostatic repulsive forces exerted by the anionic polysaccharides was evident by a significant decrease in peak intensity relative to the CPI solutions (Figure 4.11), a trend that was more pronounced in the presence of  $\iota$ -carrageenan due to the more electronegative sulphates. Furthermore, the repulsive nature of these interactions led to a shift in the initial rise in optical density to lower pH, relative to the CPI solutions (Figures 4.11 and 4.12). Critical pHs corresponding to the formation of soluble ( $\text{pH}_c$ ) and insoluble ( $\text{pH}_{\phi 1}$ ) complexes for CPI-alginate and CPI- $\iota$ -carrageenan as a function of biopolymer mixing ratios is presented in Figure 4.13. At pHs greater than  $\text{pH}_c$ , biopolymer mixtures were considered to be co-soluble, and non-interacting. For both mixtures,  $\text{pH}_c$  and  $\text{pH}_{\phi 1}$  shifted to higher pHs, as the mixing ratios increased from 5:1 to 20:1, and then reached a plateau as ratios increased to 50:1. At pH less than  $\text{pH}_{\phi 1}$ , formed complexes began to precipitate out of solution. In addition, both  $\text{pH}_c$  and  $\text{pH}_{\phi 1}$  were found to occur at higher pH values for CPI- $\iota$ -carrageenan than CPI-alginate at all biopolymer mixing ratios (Figure 4.13). As an example, for the 20:1 mixing ratio,  $\text{pH}_c$  and  $\text{pH}_{\phi 1}$  were  $6.61 \pm 0.14$  and  $5.72 \pm 0.08$ , respectively for CPI- $\iota$ -carrageenan and,  $6.02 \pm 0.11$  and  $4.80 \pm 0.02$ , respectively for CPI-alginate.

A dependence of  $\text{pH}_c$  on biopolymer mixing ratio during complexation has been previously reported by Liu et al. (2009) for pea protein isolate-GA mixtures at ratios less than 4:1, and Singh et al. (2007) for type-A and type-B gelatin with agar at ratios less than 2:1. The authors attributed the observed rise and plateau of  $\text{pH}_c$  and  $\text{pH}_{\phi 1}$  to the progressive growth of protein-protein aggregates with increasing biopolymer ratio until a critical size was reached, after which a steady state occurred. The mixing ratio dependence of  $\text{pH}_c$  highlights the importance of CPI aggregates in the complexation process, and suggests that polysaccharide chains are most likely interacting with small CPI-CPI aggregates rather than individual proteins. The presence of CPI-CPI aggregates is expected based on the high optical densities observed for CPI solutions (Figure 4.11). In non-aggregated milk protein systems, an opposite trend has been reported where  $\text{pH}_c$  was independent of mixing ratio, which was attributed to the binding of a polysaccharide to a single protein rather than an aggregate (Weinbreck et al., 2003b,

2004a; Girard et al., 2004). The dependence of  $pH_{\phi 1}$  on mixing ratio had been previously reported by Liu et al. (2009) and Weinbreck et al. (2003b, 2004a), where values shifted to more basic pH with increasing mixing ratio up to a critical value, followed by a plateau. Authors attributed this behaviour to the greater amount of protein molecules available per polysaccharide chain for binding.

Typically, proteins take on a positive net charge as solvent pH is lowered below its pI, and interact more strongly with the anionic polysaccharides. However, with highly charged polysaccharides, complexation can occur at a pH greater than the pI, which are attributed to strong electrostatic attraction to hydrophilic patches on the protein's surface (Dickinson, 1998; de Vries et al., 2003; Weinbreck et al., 2004a). This phenomenon has been previously reported for a whey protein-carrageenan (Weinbreck et al., 2004a) mixture. In the present study, the pI of CPI was found to be 5.47 (zeta potential = 0 mV) (Figure 4.14), which means with the exception of the 1:1 CPI- $\iota$ -carrageenan system, and the 1:1 and 10:1 CPI-alginate systems, all mixing ratios initiated complex formation (i.e., at  $pH_c$ ) when both biopolymers carried a similar net negative charge. This effect was more pronounced when CPI was mixed with  $\iota$ -carrageenan. CPI- $\iota$ -carrageenan systems were also found to form insoluble complexes (i.e., at  $pH_{\phi 1}$ ) at a pH greater than the pI, whereas  $pH_{\phi 1}$  occurred at  $pH < pI$  for CPI-alginate. Dickinson (1998) and Doublier et al. (2000) reported that  $-OSO_3^-$  groups have greater attraction to  $-N^+R_3$  groups on the protein's surface than  $-COO^-$  groups, which may be attributed to earlier complexation in solution.

Electrophoretic mobility measurements for a 20:1 CPI-polysaccharide mixture and, corresponding CPI, alginate and  $\iota$ -carrageenan solutions as a function of pH are given in Figure 4.14. In contrast to CPI, which had a pI value of 5.47, polysaccharide solutions carried a net negative charge over the entire pH range. Similar zeta potential values have been reported for alginate by Duce et al. (2004) and Harnsilawat et al. (2006) as a function of pH. In the case of the CPI- $\iota$ -carrageenan mixture the formation of both soluble ( $pH_c = 6.61$ ) and insoluble ( $pH_{\phi 1} = 5.72$ ) complexes occurred under conditions where all biopolymers carried a net negative charge (Figure 4.14). Whereas, in the CPI-alginate mixture, soluble complexes formed ( $pH_c = 6.02$ ) under conditions where both biopolymers carried a net negative charge. However the formation of



insoluble complexes ( $\text{pH}_{\phi 1} = 4.80$ ) occurred at pH less than 5.70 where CPI carried a net positive charge (Figure 4.14).

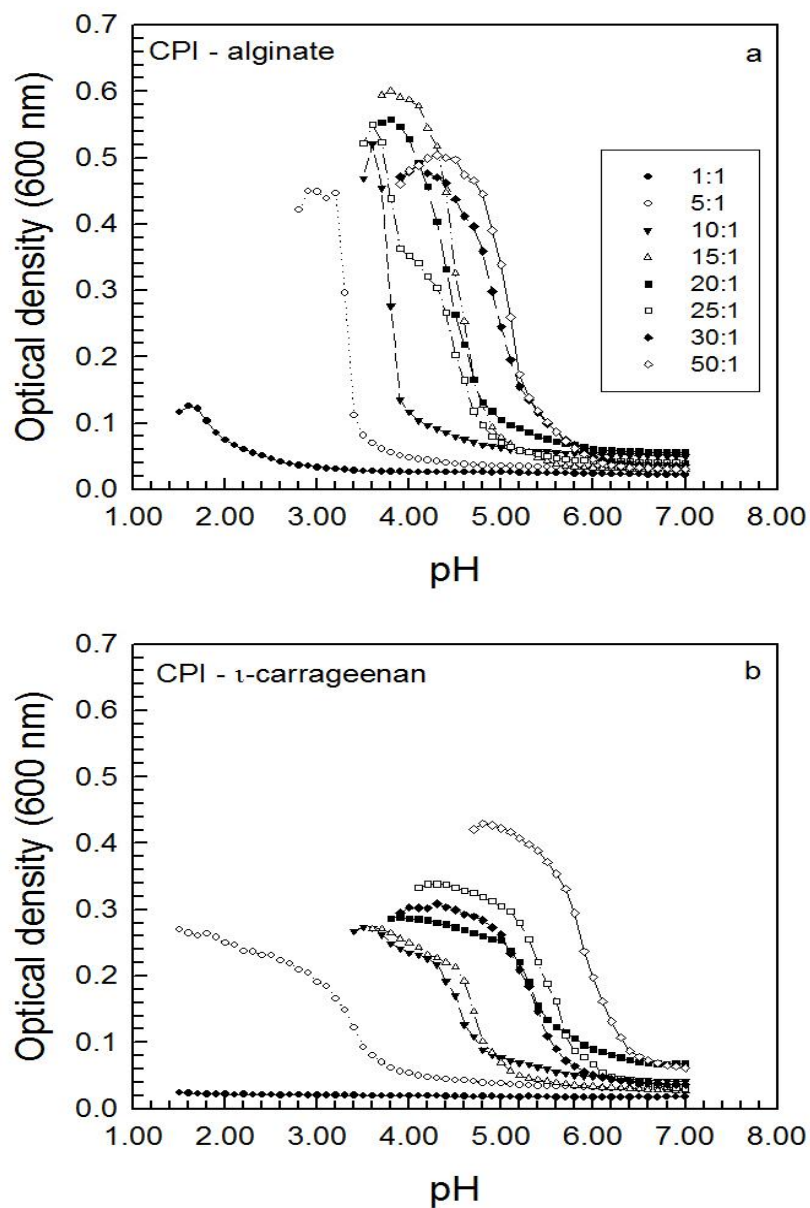


Figure 4.12. Mean optical density curves for mixtures of CPI-alginate (a) and CPI- $\iota$ -carrageenan (b) as a function of pH and biopolymer mixing ratio (% w/w) ( $n = 3$ ).

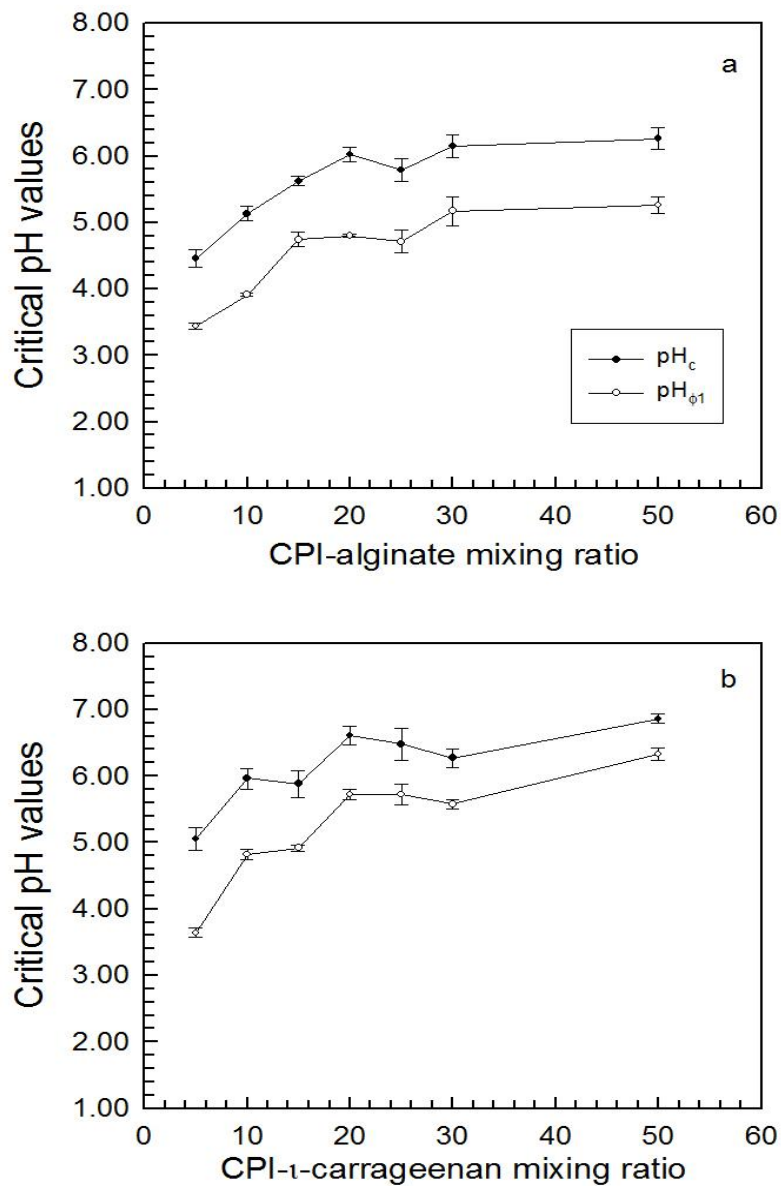


Figure 4.13. Mean critical pH values associated with the formation of soluble (pH<sub>c</sub>) and insoluble (pH<sub>φ1</sub>) complexes as a function of biopolymer mixing ratio for CPI-alginate (a) and CPI-ι-carrageenan (b) mixtures. Data points represent the mean  $\pm$  one standard deviation, n = 3).

Electrophoretic mobility measurements for both CPI-polysaccharide mixtures estimated a net neutral surface charge of formed complexes at pH 4.55, which is

presumed to reflect the optimal stoichiometric equivalent of positive reactive sites associated with CPI and the negative reactive sites on each polysaccharide. Similar results were found while determining the optimal biopolymer mixing ratios during optical density measurements (Figure 4.14). For pea protein isolate-GA, Liu et al. (2009) reported this pH to correspond to  $pH_{opt}$  within the optical density spectrum. Although  $pH_{opt}$  values were not reported in the current study (due to uncertainties surrounding pH-dependent precipitation), maximum optical density was found for the CPI-polysaccharide mixtures within the optical density-pH scans close to pH 4.00 (Figure 4.12). A shift in the pH of net neutrality to lower pH upon complexation relative to the protein alone has been reported by Harnsilawat et al. (2006) for  $\beta$ -lactoglobulin-sodium alginate and Schmitt et al. (1999) for  $\beta$ -lactoglobulin-GA. These findings suggest that the reactive group present on the polysaccharide backbone has a direct impact on both surface charge of the formed complexes and the initial protein-polysaccharide interactions.

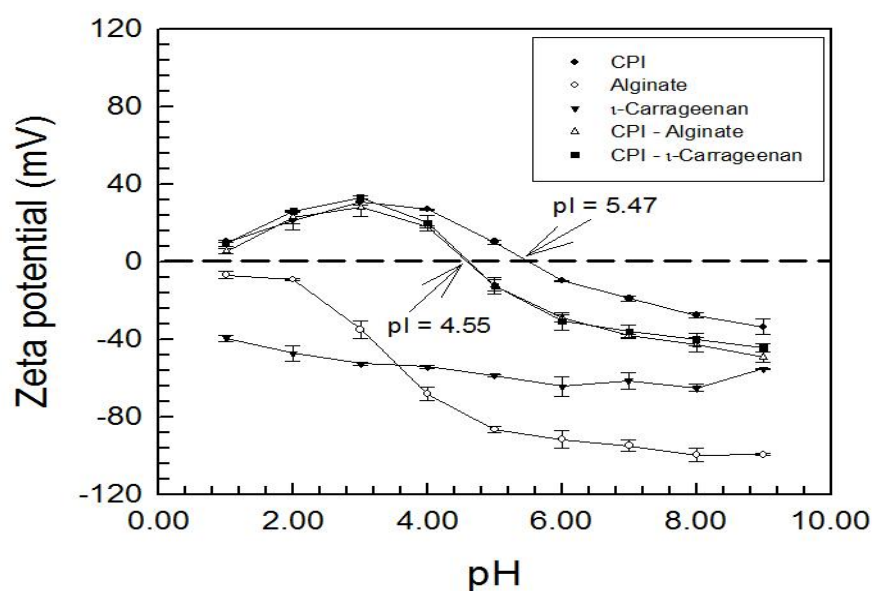


Figure 4.14. Mean zeta potential (mV) measurements as a function of pH for CPI, alginate and  $\iota$ -carrageenan, and CPI-alginate and CPI- $\iota$ -carrageenan (20:1 mixing ratio) biopolymer systems. Data represents the mean  $\pm$  one standard deviation,  $n=3$ ).

#### 4.2.4 Nature of CPI-polysaccharide interactions

In general, complex coacervation is driven by electrostatic attractive forces between proteins and polysaccharides of opposing charge, where the role of hydrogen bonding and/or hydrophobic interactions in the process is less well understood. Due to difficulties in delineating contributions from each intermolecular force/interaction, solvent manipulation is typically used as a means to elucidate these contributions (Kaibara et al., 2000; Weinbreck et al., 2004a; Uruakpa and Arntfield, 2005). In the present study, the nature of CPI-alginate and CPI- $\iota$ -carrageenan interactions were investigated in the presence of destabilizing agents (urea and NaCl) and as a function of temperature (21°C vs. 60°C). Changes to optical density profiles (and critical pH values) were used to determine the significance of each force/interaction in complex coacervate formation.

The effect of added NaCl (0.1 M) on complex formation within CPI-alginate and CPI- $\iota$ -carrageenan mixtures is given in Figure 4.15. For both systems, Na<sup>+</sup> and Cl<sup>-</sup> ions acted to screen reactive sites at the biopolymer's surface, significantly inhibiting complex formation. With the addition of NaCl, optical density curves shifted to higher pHs relative to systems without. The initial rise in optical density for both CPI-polysaccharide mixtures closely resembles those of CPI solutions shown in Figure 4.11. The rise in optical density shown in Figure 4.15 reflects an increased amount of CPI-CPI aggregation. Liu et al (2010a) observed that the addition of 0.1 M NaCl disrupted electrostatic attraction (and complex formation) between a pea protein isolate and GA and resulted in a higher degree of protein-protein aggregation.

Complex formation in mixtures of CPI-alginate and CPI- $\iota$ -carrageenan was investigated in the presence of 0.1 M urea by turbidimetric analysis relative to a control (without urea). The addition of urea, acts to disrupt both hydrogen bonding and hydrophobic interactions within the system (Uruakpa and Arntfield, 2006). For CPI-alginate mixtures, the addition of urea acted to shift both pH<sub>c</sub> and pH<sub>01</sub> from  $6.02 \pm 0.11$  and  $4.80 \pm 0.02$ , respectively (control) to  $5.26 \pm 0.07$  ( $p < 0.05$ ) and  $4.46 \pm 0.04$  ( $p < 0.05$ ), indicating that hydrogen bonding played a secondary role in CPI-alginate interactions (Figure 4.16a). Similar findings were found for CPI- $\iota$ -carrageenan mixtures where critical pH values shifted towards lower pHs relative to the control: (pH<sub>c</sub>)  $6.61 \pm 0.14$

to  $6.25 \pm 0.14$  ( $p < 0.05$ ) and, ( $pH_{\phi 1}$ )  $5.72 \pm 0.08$  to  $5.31 \pm 0.15$  ( $p < 0.05$ ), respectively (Figure 4.16b). Liu et al (2010a) reported a similar shift in critical pH values with the addition of urea in pea protein isolate-GA mixtures. Secondary roles of hydrogen bonding in the complexation of proteins and polysaccharides have been previously reported for mixtures of xanthan gum-gelatin (Lii et al., 2002), alfalfa rubisco-pectin (Antonov and Sochinsky, 2000) and  $\beta$ -lactoglobulin-pectin (Girard et al., 2002).

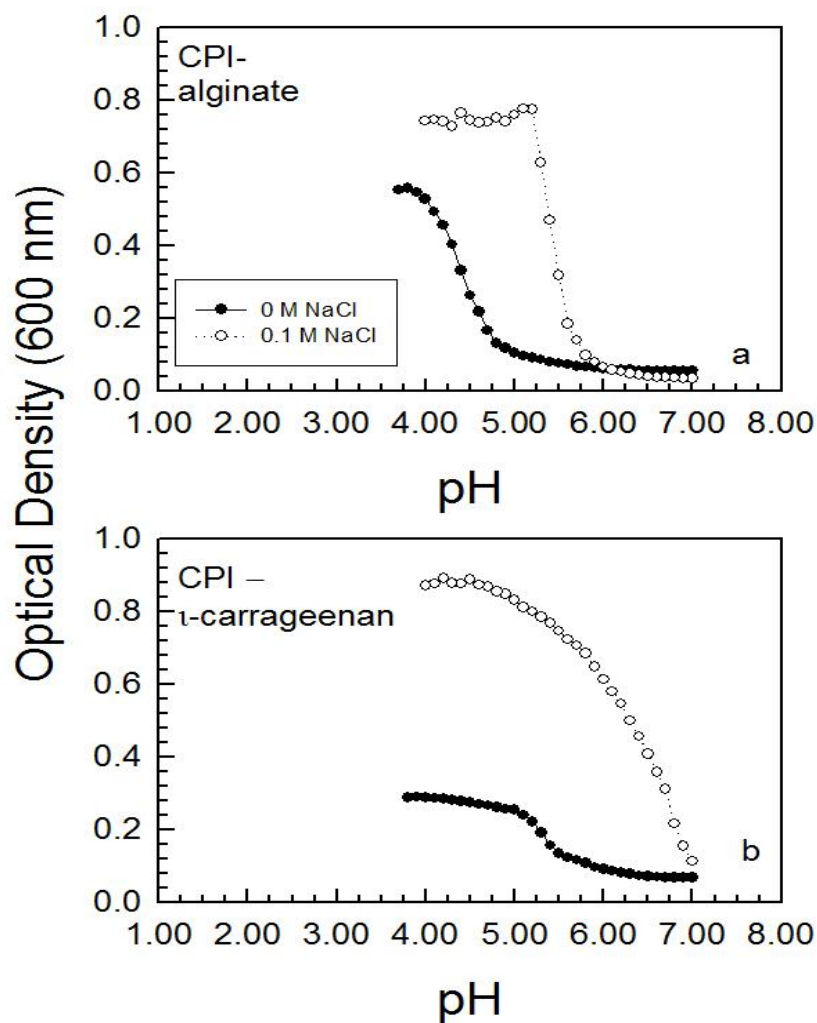


Figure 4.15. Mean optical density curves for mixtures of CPI-alginate (a) and CPI- $\iota$ -carrageenan (b) as a function of pH, in the presence and absence of 0.1 M NaCl (20:1 CPI:polysaccharide; w/w) ( $n = 3$ ).

Figure 4.16 also shows the effect of temperature (60°C vs. 21°C (control)) on complex formation in CPI-alginate and CPI- $\iota$ -carrageenan by turbidimetric analysis. For CPI-alginate and CPI- $\iota$ -carrageenan, raising the temperature to 60°C did not shift critical pH values significantly from the control suggesting that initial complex formation was not promoted by hydrophobic interactions ( $p > 0.05$ ) (Figure 4.17a,b). The lowering of  $\text{pH}_{\phi 1}$  is thought to reflect the breaking of hydrogen bonding. If hydrophobic interactions were to have a stabilization role during complex formation, a shift to higher pHs would be expected. Temperature independence of critical pH values has been reported by Weinbreck et al. (2004a) for whey protein-carrageenan and by Kaibara et al. (2000) for BSA-poly(dimethyldiallylammonium chloride), supporting the present findings that hydrophobic interaction have a limited role in complex formation. However, since CPI-polysaccharides in the present study are presumed to involve interactions between polysaccharides and CPI-CPI aggregates rather than individual proteins, hydrophobic interactions may have a role in stabilizing the complex structure once formed. Although the structures of the precipitate were not characterized, it was observed visually that their sizes increased substantially at lower pH values (1.5-2.0). Liu et al. (2010a) highlighted the possible role of hydrophobic interactions in stabilizing complex structures at low pH values (pH 3.10), rather than being important for complex formation. Liu et al. (2010a) found that these interactions were more pronounced at 60°C resulting in larger formed complexes as confirmed by confocal scanning laser microscopy.

#### **4.2.5 Coacervation at a higher total biopolymer concentration**

Protein and carbohydrate content of the CPI-alginate and CPI- $\iota$ -carrageenan systems were determined at a high total biopolymer concentration (1.0% w/w) to ensure that the mixed systems were undergoing complexation. Analysis revealed that ~99% of the CPI, ~85% of the  $\iota$ -carrageenan and ~78% of the alginate was associated with the pellet, with the remaining concentrations found in the supernatant. These findings indicate that associative phase behaviour was still occurring when the total biopolymer concentration was raised from 0.05% to 1.0% (w/w). In contrast, the CPI blank contained 44.5% and 55.5% of protein in the pellet and supernatant, respectively. In the

case of the alginate and ι-carrageenan solutions, no phase separation occurred and these polysaccharides were distributed throughout the entire solution.

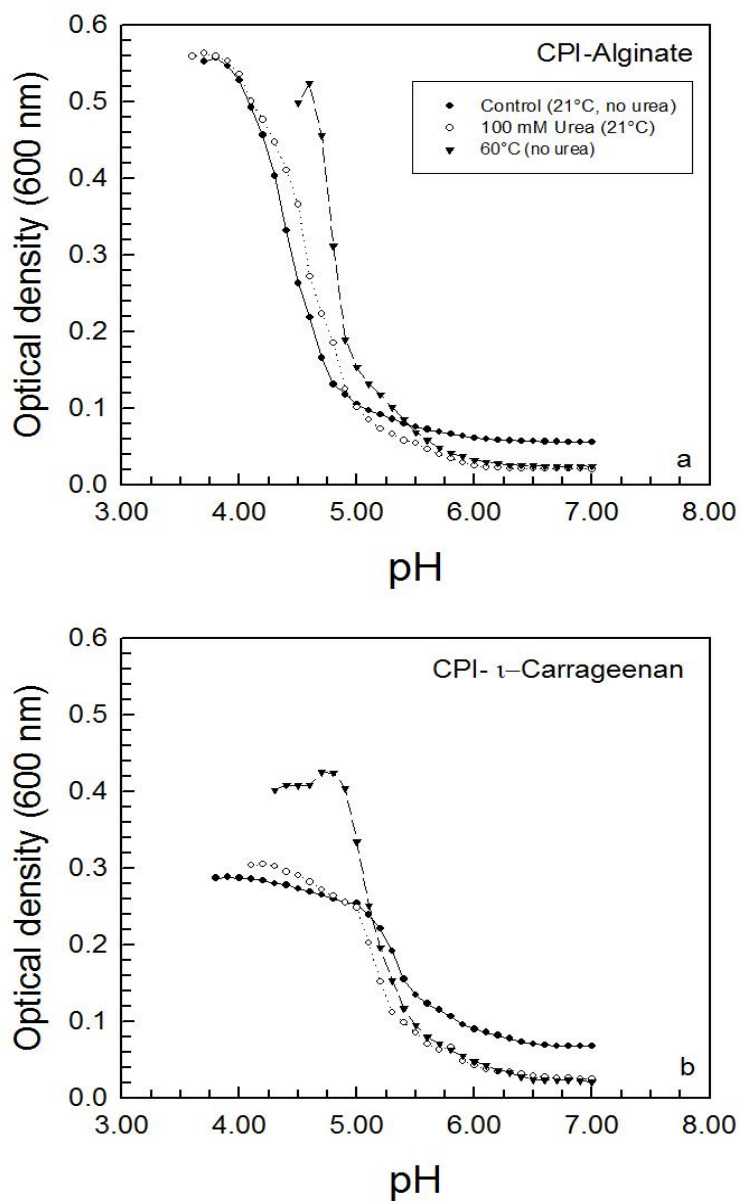


Figure 4.16. Mean optical density curves for mixtures of CPI-alginate (a) and CPI-ι-carrageenan (b) as a function of pH, temperature (21°C vs. 60°C, without urea) and urea (at 21°C) (20:1 CPI:polysaccharide) (n = 3).

#### 4.2.6 Solubility

The solubility of CPI, and CPI-alginate and CPI- $\iota$ -carrageenan solutions were investigated at pH values corresponding to conditions where the biopolymers were considered to be: a) co-soluble (pH greater than  $\text{pH}_c$ ); b) present in the form of soluble complexes ( $\text{pH}_c$  less than pH greater than  $\text{pH}_{\phi 1}$ ; at 2 different pHs); or c) present as insoluble complexes (pH less than  $\text{pH}_{\phi 1}$ ). Solubility of the CPI solution showed a typical U-shaped curve showing minimal solubility (5.47%) at a pH of 5.41 (Figure 4.17), which corresponded to a pH where the protein was electrically neutral (pH 5.47, Zeta potential = 0 mV, Figure 4.14). Maximum solubility was seen at the pH extremes tested, where at pH 7.00, 76.58% of the protein was soluble, compared with 53.32% at pH 4.50. Findings were comparable to that of Paulson and Tung (1987) who found the solubility minimum for a laboratory prepared CPI of pH 5.0. Similar percent solubility for CPI extracts from a variety of canola meals (56-66%) as in the present study were found by Aluko and McIntosh (2001).

Protein solubility was high (65-85%) in both the CPI and the mixed CPI-polysaccharide systems pH 7.00 (pH greater than  $\text{pH}_c$ ) (Figure 4.17). The solubility of CPI was enhanced significantly at this pH in the presence of alginate ( $p < 0.05$ ), whereas, in the presence of  $\iota$ -carrageenan a decrease was seen ( $p > 0.05$ ). The effect of alginate on CPI solubilities may be due to its higher surface charge (Zeta potential = -95.0 mV) when compared to  $\iota$ -carrageenan (Zeta potential = -61.5 mV) at pH 7.00 (Figure 4.14). Under complexation conditions, percent solubility remained relatively high (greater than 65%) when CPI-alginate complexes were present at pHs greater than  $\text{pH}_{\phi 1}$ , and represented a substantial improvement over CPI alone at pHs corresponding to its pI (pH. 5.47) (Figure 4.17). At pH less than  $\text{pH}_{\phi 1}$ , the presence of CPI-alginate complexes resulted in a complete loss in solubility and the system was dominated by precipitates. In contrast, a similar solubility trend was found between CPI- $\iota$ -carrageenan mixtures and CPI solutions, except the solubility of the mixed system did not to increase at pH 4.50 as did CPI alone (Figure 4.17). Differences in solubility between CPI-alginate and CPI- $\iota$ -carrageenan are thought to be related to polysaccharide surface charge (alginate



was greater than ι-carrageenan over the pH range examined) and, the greater tendency for ι-carrageenan to induce CPI precipitation.

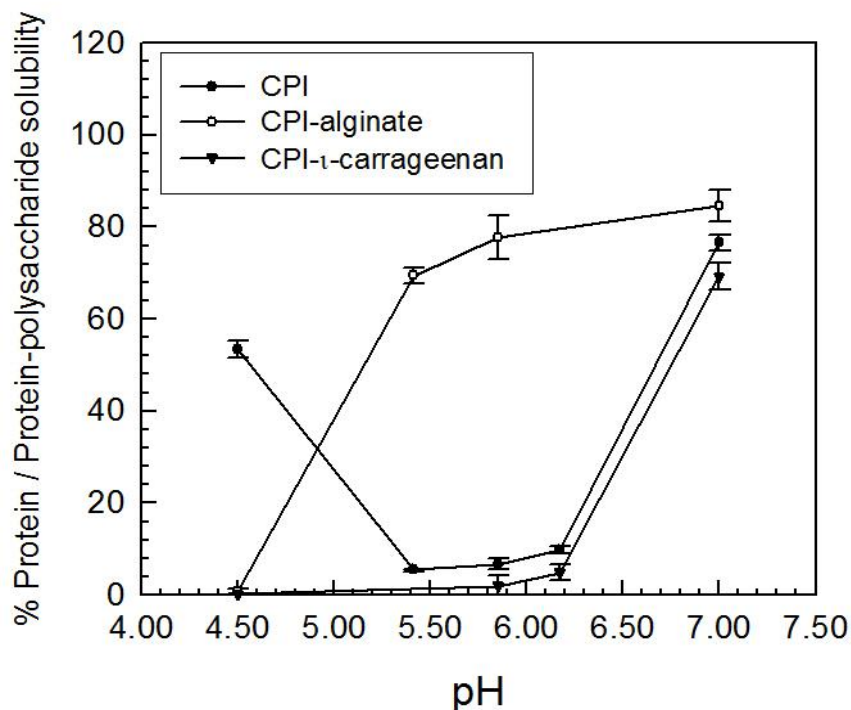


Figure 4.17. Solubility as a function of pH for CPI, CPI-alginate and CPI-ι-carrageenan (20:1 CPI:polysaccharide) solutions. Data points represent the mean  $\pm$  one standard deviation,  $n = 3$ ).

#### 4.2.7 Summary

Complex formation between CPI and both alginate and ι-carrageenan were found to be dependent on biopolymer mixing ratio (less than 20:1), above which complexation was found to be independent of biopolymer ratio. Complexation was thought to involve small CPI aggregates with polysaccharide chains rather than individual proteins due to the overlap seen between the mixed and CPI optical density profiles and the biopolymer dependence of critical pH values. In general, complex formation occurred at pH greater than the pI of the CPI, where both biopolymers carried a net negative charge, an effect more pronounced in the presence of the sulphated polysaccharide, ι-carrageenan. This effect was thought to be due to the highly

electronegative reactive sites on the polysaccharides, which interacted with positively charged patches on the protein surface. The nature of interactions involved with complexation of these biopolymers was found to be driven primarily by electrostatic attractive forces with secondary stabilization by hydrogen bonding. Solubility of the mixed CPI-alginate system showed a marked improvement over CPI alone and CPI- $\iota$ -carrageenan as a function of pH.

## CHAPTER 5.0 GENERAL CONCLUSIONS

The overall goal of this thesis was to better understand mechanisms governing complex coacervation (or associative phase separation) between plant proteins and anionic polysaccharides, so that the structure and functional attributes of the formed complexes could be controlled, and later tailored for specific applications in the food industry. Although complex coacervation has been well documented involving animal proteins with anionic polysaccharides, such as bovine serum albumin-polydiallyl dimethyl ammonium chloride (Wen and Dubin, 1997), casein-sodium carboxymethyl cellulose (Syrbe, 1998),  $\beta$ -lactoglobulin-alginate (Harnsilawat et al., 2006) or whey-GA (Weinbreck et al., 2003a), with mechanisms recently reviewed by Turgeon et al. (2007); few studies have focused on understanding structure-function relationships involving plant protein-polysaccharide interactions. The present research: a) builds upon literature studying mechanisms governing complex coacervation involving pea proteins and GA; b) initiated research on structure-function relationship involving mixtures of canola proteins with sulphated ( $\iota$ -carrageenan) and carboxylated (alginate) polysaccharides; and c) discusses similarities and differences between animal and plant proteins for the role in the coacervation process.

### *a) Associative phase behaviour in admixtures of pea proteins and GA.*

In earlier work by Liu et al. (2009, 2010a), the associative behaviour in admixtures of a pea protein isolate-GA systems were investigated as a function of pH, salt content and biopolymer mixing ratio. The authors found optimal conditions to occur at a protein: polysaccharide mixing ratio of 2:1 in the absence of added salts and at pH 3.60. The authors also found that the formation of soluble and insoluble complexes (and associated critical pHs) were dependent on the biopolymer mixing ratios less than 4:1, and that coacervate formation was primarily driven by electrostatic attractive forces with secondary stabilization by hydrogen bonding. Complexes were thought to form through

the interactions between pea protein aggregates and GA molecules rather than individual proteins, and that hydrophobic interactions played a role in complex stability at acidic pH ( $\sim 3.10$ ) conditions, but not with complex formation. However, the authors stopped short of delineating the individual role of the major globulin proteins, Lg and Vn in the behaviour of their isolate.

In the present research (see section 4.1), the effects of these proteins relative to a mixed isolate on complex formation with GA were examined. According to a classification discussed by de Kruif et al. (2004), GA would be considered a weakly charged polyelectrolyte based on carboxyl reactive group. In the present study, mixtures of GA with crude isolates produced by salt/pH extraction (e.g, mixed isolate, Lg and Vn) and enriched isolates produced by subsequent LPLC purification (e.g., Lg and Vn) under optimal conditions described by Liu and co-workers (2009) were examined. In all cases complex formation occurred over a narrow pH range where biopolymers exhibited a similar net opposite charge (at pHs less than the pI). Complexes were thought to form between protein-protein aggregates and GA this is based on isolate optical density profiles which suggested aggregates were present at  $pH_c$  values corresponding to mixed systems. Similar to Liu et al. (2009, 2010a), crude protein-GA systems and the enriched Lg-GA mixtures showed a significant shoulder in its optical density pH-titration profile at  $pH \sim 3.00$  indicating that complexes were more stable most likely due to hydrophobic stabilizing interactions occurring within the protein-protein aggregate participating in the complex.

Overall, the coacervation behaviour of crude and enriched Lg-GA and Vn-GA systems were comparable to the crude mixed isolate-GA system, with minor differences found in the enriched Vn-GA and Lg-GA systems relative to the crude as it relates to its formation of soluble complexes only. The Vn-GA system also switched from a system where complexes remained suspended to one that precipitated at pHs less than  $pH_{\phi 1}$  upon enrichment, however further investigation would be required to identify reasons for behavioural differences. Investigation of changes to the protein conformation of the enriched Lg and Vn upon complexation with GA indicated that only the enriched Vn experienced a conformational change. In general solubility profiles for the mixed systems were not found to improve in comparison to the protein isolates alone. The

solubility profiles suggest that there would be little or no benefit to separation of the mixed isolate into crude Lg and Vn isolates, in the development of formulated functional protein-based ingredients.

*b) Associative phase behaviour in admixtures of CPI and carboxylated and sulphated polysaccharides.*

Similar coacervation mechanisms were also apparent for a CPI system when mixed with anionic polysaccharides, suggesting similarities between legume and oilseed globulin-type proteins (see section 4.2). In this case, complexation of CPI with both a weakly (carboxylated alginate) and strongly (sulphated ι-carrageenan) charged polyelectrolyte were investigated as a function of pH and biopolymer mixing ratio. In all cases, the addition of either polysaccharide acted to shift structure-forming events to lower pH and reduced the magnitude of optical density relative to the CPI, this is presumed to be attributed to an increased amount of electrostatic repulsive forces arising between polysaccharides in solution; an effect greater for ι-carrageenan than alginate. Similar to the pea protein systems, complex formation was thought to occur between protein-protein aggregates rather than individual proteins. In addition, a similar trend for biopolymer mixing ratio dependence was found between CPI-polysaccharide mixtures and the pea protein isolate-GA system reported by Liu et al. (2009) for both the formation of soluble and insoluble complexes.

In general, complex formation occurred at a pH greater than the pI of the CPI, where both biopolymers carried a net negative charge, an effect more pronounced in the presence of the sulphated polysaccharide, ι-carrageenan. This effect was thought to be caused by the highly electronegative reactive sites on the polysaccharides, which interacted with the positively charged patches on the protein's surface. The nature of interactions involved with complexation of these biopolymers was found to be driven by electrostatic attractive forces with secondary stabilization by hydrogen bonding. However, unlike the crude pea protein isolates (mixed, Lg and Vn)-GA and enriched Lg-GA systems (See Section 4.1), precipitation occurred at pH less than  $pH_{\phi 1}$  rather than remaining in suspension as coacervates. Based on de Kruif et al. (2004), systems that form precipitates versus coacervates seem highly dependent upon the strength of

interactions between the two biopolymers. Thus, in the case of both CPI-polysaccharide systems, biopolymer interactions between the two were considered strong, despite alginate being classified as a weakly charged polyelectrolyte. Alginate was thought to induce precipitate formation due to its higher charge density than GA, and based on differences in surface characteristics of the two proteins.

Solubility of the CPI-alginate system showed a marked improvement over CPI alone and CPI- $\iota$ -carrageenan system as a function of pH. Findings suggest a CPI-alginate mixture may have potential for creating a formulated plant protein-based ingredient for specific food and biomaterial applications, which would have improved functionality over CPI alone. In the present study, the CPI- $\iota$ -carrageenan was considered a very strong complexing system due to the presence of sulphate reactive sites along the polysaccharide backbone to induce relatively fast precipitation at a set pH; reflected by poor solubility once bound to the CPI. In contrast, CPI-alginate complexes, despite the strong attractive interactions between the two biopolymers, tended to remain suspended in solution longer than the CPI- $\iota$ -carrageenan complexes (based on visual observations and erratic optical density readings at pHs near or after the maximum). The latter enables for improved functionality over a greater pH range than either CPI alone or CPI- $\iota$ -carrageenan systems. A further mechanistic understanding is needed to better understand the reasons governing improved solubility, but may be related to shifts in the protein conformation upon complexation.

*c) Similarities and differences in associative phase behaviour involving admixtures of anionic polysaccharides with plant and animal proteins.*

In general, mechanisms governing associative phase behaviour within plant proteins-polysaccharide mixtures are similar to that of animal protein-polysaccharide mixtures. For instances, complexation in both systems (i.e., those with animal or plant proteins) occur over a narrow pH range, and typically occurs when biopolymers carry an opposing net charge (i.e., at pH less than the pI of the protein) when weakly charged polyelectrolytes (e.g., GA) are present (see section 4.1) (Weinbreck et al., 2003a, 2004b; Liu et al., 2009), and may complex when biopolymers carry a similar net charge if strong polyelectrolytes are present (e.g., carrageenan) (see section 4.2) (Weinbreck et

al., 2003b, 2004a; de Kruif et al., 2004). In the case of the latter, negatively charged polysaccharides are attracted to positively charged patches on the protein's surface (Schmitt et al., 1998). Complexation follows 2-main structure forming events, associated with the formation of soluble and insoluble complexes, leading to either coacervate or precipitate formation depending on the level of biopolymer interactions occurring (Schmitt et al., 1998; Turgeon et al., 2003). Complexes form primarily via electrostatic attractive forces with secondary stabilization by hydrogen bonding (Girard et al., 2002; Liu et al., 2010a). However, in the case of plant protein-polysaccharide mixtures, the role and size of protein-protein aggregates seems to play a more significant role in complex formation and stability than in the case of animal protein-polysaccharide systems (Liu et al., 2009, 2010a). In the case of the former, complexes are thought to be comprised of larger protein-protein aggregates and individual polysaccharide molecules leading to a biopolymer mixing ratio dependence of  $pH_c$ . Under acidic pH conditions, hydrophobic interactions involved with stabilizing the protein-protein aggregates seem to provide extra stability to the complex structure (Liu et al., 2010a). In general, no pH-dependence of  $pH_c$  occurs in mixtures of animal proteins and polysaccharides, as the latter tends to interact with individual proteins (Weinbreck et al., 2003b). Under acidic conditions, complexes appear less stable than their plant protein alternatives possibly the result of lower amounts of hydrophobic interactions occurring.

*d) Overall thesis summary.*

Research findings from this work validated the central hypothesis of the thesis (i.e., *'by controlling the coacervation process and resulting structures, the functional attributes of both pea and canola proteins can be improved'*). Where it was found that biopolymer-type (protein and polysaccharide), mixing ratio and pH all influenced the complexation process and resulting structures. Although not all conditions in the present study led to improved functional attributes, the CPI-alginate system showed improved solubility over CPI alone - highlighting the potential of this technology for developing formulated protein-based ingredients for the food and biomaterial industries. This complemented Liu et al. (2010b) who studied pea protein isolate-GA mixtures to find improved functional attributes (e.g., emulsion and foaming stability) over pea proteins

alone. Findings from this study also led to a better mechanistic understanding of the coacervation process involving admixtures of plant proteins and anionic polysaccharides.



## CHAPTER 6.0 FUTURE STUDIES

The interaction of various pea protein isolates and GA was studied with a focus of determining differences between each isolate during complexation. Fluorescence intensity revealed an increase in intensity upon complexation of enriched Vn with GA, further studies could provide greater detail as to the changes are occurring. Circular dichroism or Raman microspectroscopy could provide information on changes to the secondary structure ( $\beta$ -sheets,  $\alpha$ -helices and random coils). To produce formulated protein-based ingredients it is crucial to investigate various protein and polysaccharide mixtures. Investigating structural changes of these different systems would allow for greater understanding of structure-function mechanisms of the formed complexes.

For pea protein isolate-GA systems no improvement in solubility was seen when compared with protein isolate systems. Investigating other polysaccharides (e.g., chitosan, alginate, carrageenan and gellan gum) may lead to systems which show improvement in functional properties when compared to pea protein alone allowing for the creation of formulated pea protein ingredient systems.

This thesis investigated using a canola protein isolate as a potential food ingredient. Two polysaccharides (alginate and  $\kappa$ -carrageenan) were focused on. These polysaccharides are known to be sensitive to calcium (especially for gel formation) further studies investigating the effect of calcium on complex formation, aggregation and structure-forming abilities would provide further insight into possible applications for these materials.

Solubility is an important functional attribute as protein solubility influences other functional properties (e.g., emulsifying, gelation and foaming). Solubility curves for the CPI-alginate system showed improvement over that of CPI- $\kappa$ -carrageenan system and CPI alone. This improved solubility may indicate a possible application of CPI-alginate system in creating plant protein-based ingredient. An investigation of other functional properties (e.g., gelation, emulsifying and foaming) will provide further

information on the viability of a CPI-alginate food ingredient. Further studies using other polysaccharides (e.g., GA, gellan gum and chitosan) may also be explored to determine different system which could be tailored plant-protein ingredients.

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